ANNUAL REVIEW OF MICROBIOLOGY

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VOLUME III

1949

ANNUAL REVIEWS, INC.
STANFORD, CALIFORNIA

ANNUAL REVIEWS, INC.
STANFORD, CALIFORNIA

FOREIGN AGENCIES

London:
H. K. Lewis & Company, Limited
136 Gower Street, London, W.C. 1

Moscow:
Mezhdunarodnaya Kniga
Kuznetsky Most, 18

The Hague:
Martinus Nijhoff
9, Lange Voorhout

PRINTED AND BOUND IN THE UNITED STATES OF AMERICA BY
GEORGE BANTA PUBLISHING COMPANY

PREFACE

The cordial reception accorded this *Review* indicates that it is contributing significantly to the assimilation of the evergrowing literature on microbiology. At the same time it reflects the fact that readers, and indeed the Editors and Editorial Committee, appreciate the effort expended by the reviewers. Once again we express our gratitude to all who have contributed to the authorship of the present volume.

It is a pleasure, as in past years, to extend our thanks to the editorial assistants of Annual Reviews, Inc., and to the George Banta Publishing Co. for their painstaking care and hearty cooperation in the preparation of the present issue.

W.H.T.	C.B. van N.
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E.C.S.	

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BACTERIAL VARIATION[1,2]

By Joshua Lederberg
Department of Genetics, University of Wisconsin, Madison, Wisconsin

As far as consistent with an integrated presentation, this article will exclude literature discussed in three earlier reviews (5, 52, 61) with whose viewpoint the writer concurs. In particular, the exposition of the problem of directed adaptive variation has been fully discussed. Spontaneous mutation and natural selection are adequate to account for most adaptive changes in bacterial populations. From a general biological standpoint, exceptions to this rule would be very instructive, but except for induced lysogenicity, claims of lamarckian responses in bacteria have not been sufficiently fortified by experiment.

The classification of bacterial variations as mutations can be made only tentatively except where genetic analysis permits. Unfortunately, this is now possible only in strain K-12 of *Escherichia coli* which shows genetic recombination. A number of other mechanisms of heritable variations, summarized in Table I, are familiar to geneticists, and others may yet be established. Nevertheless, most of our information on bacterial variation is most conveniently organized in terms of mutation.

TABLE I

Categories of Genetic Variation

A. Changes which may occur in single cell cultures
   1. Mutation—chemical change or destruction of a hereditary particle
   2. Attenuation—quantitative changes in extranuclear units
   3. Segregation—from a heterokaryon or a heterozygote
   4. Chromosomal rearrangement and polyploidy
B. Changes involving extrinsic biological units
   5. Fusion of cells leading to heterokaryon or heterozygote
   6. Infection and transformation

Bacterial Mutation Studies

Phage resistance mutants.—Quantitative mutation studies are easiest when the mutants can be counted by inspection. Mutations

[1] This review covers the period to February, 1949.

[2] Paper No. 394 of the Department of Genetics, College of Agriculture, University of Wisconsin, Madison, Wisconsin.

for resistance to bacteriophages have many virtues, as the number of resistant mutants in a population is estimated by plate counts of the survivors of phage attack. Infection and lysis of the cell is an all-or-none affair, and under appropriate conditions, every sensitive cell is destroyed, and every resistant survives. Account must be taken, however, of genetic variations in the phage stocks used (6).

In their classical work, Luria & Delbrück (62) found enormous fluctuations in the numbers of mutants in replicate cultures and showed that this argued against the direct induction of the mutations by the phage. This very high variance, compared to the sampling errors revealed by replicate assays from the same culture, shows that some factor not under experimental control is responsible for the fluctuations, and therefore for the mutations. That is to say, the mutations are spontaneous. The variance can be accounted for by the occurrence of the mutations throughout the development of the cultures. Those few which occur early will generate large clones of resistant cells, while the later ones will be multiplied by a smaller factor. The theoretical distributions of the mutants as a function of the mutation rate have not been published (see 28), but an approximate solution was given. With this method, the rate of mutation to resistance to T1 in *E. coli* B was estimated at 2×10^{-8} per fission cycle.

An independent estimate of the mutation rate, calculated from the fraction of cultures containing no mutants, gave 3×10^{-9} per fission cycle. A detailed investigation of this discrepancy led to the conclusion that mutant cells (first method) are produced about six times as rapidly as mutant clones (second method), which must mean that the mutant clones are initiated two or three generations before any descendant becomes phenotypically resistant. In other words, there is a variable lag between the occurrence of the genetic change and its phenotypic expression (74). Phenotypic lag may account for the fact that nondividing cultures do not accumulate mutants (62), and that disproportionate numbers of mutants appear during the first few divisions of a new growth cycle.

These rates are probably a sum for several distinct mutations. Selection for survivors of T1 attack leads to a variety of resistant types differing in nutritional requirements, colony morphology, and cross-resistance. The predominant types are /1, 5, or V_1^r, resistant both to T1 and T5, and /1, or V_{1a}^r, resistant only to T1.

Recombination tests in K-12 have proven them to be distinct mutations (50). There is some variation in the proportions of these main types in different stocks, but this may be due to epistatic effects as well as to differential mutation rates (6).

In strain B of *E. coli*, the /1 mutation is associated with a requirement for tryptophane (2), not yet observed in K-12. In a similar system, Wollman (103) has shown that tryptophane-independence can not be restored by reversion, but only by two mutational steps which leave the culture still resistant.

A third described type of resistant mutant is V_{1b}^r, an unstable mucoid form frequently found in K-12. Certain complex resistance patterns have also been described as occurring rarely (6, 23, 60). Since they can be pictured as the superposition of more frequent simpler patterns, they may not be simple gene changes, but "mass rearrangement of the genetic material of the bacterial cell, possibly comparable to that responsible for chromosomal rearrangements in higher organisms." Unfortunately, K-12 in which such a hypothesis could be tested has not yet produced such complex types.

The different resistance patterns described above probably depend on mutations at different loci. However, a type has been described, V_{1c}^r, which may be allelic to V_1^r (54). V_{1c}^r is partially resistant to T5, T1h, and T_1, increasing in that order. With V_1^r, no recombinations which would be completely sensitive were observed in 199 tests, but a diploid heterozygote which was discovered in this experiment proved to be sensitive. Thus V_1^r and V_{1c}^r are either allelic or closely linked. In any event they have a complementary action since the combination of the two mutants showed the wild, sensitive, phenotype.

Mutation to phage resistance, which occurs spontaneously, may be accelerated by the use of various mutagenic agents, preeminently radiations (24). The most striking feature of this work is an apparent delay in the expression of the mutations which are induced either by x-rays or by ultraviolet light. Phenotypic lag, as discussed for spontaneous mutations, accounts for part of this delay. In fact it is difficult to explain the induced zero-point mutations which are detectable immediately after irradiation. Radiations may have a direct accelerating effect on phenotypic lag, perhaps by destroying the residual receptors which are responsible for sensitivity. On the other hand, the induced zero-point mutations may be partly artefacts. Such mutations are produced in

appreciable numbers only by doses which kill most of the bacteria. The killed bacteria, however, are still able to absorb, but not to regenerate phage. When present in excess, they may protect sensitive bacteria from infection long enough to allow phenotypic lag to run its course. According to Beale (6), there should be a severalfold excess of phage over bacteria to ensure immediate lysis of sensitive cells, and if the sterilized bacteria are taken into account, this condition was not always fulfilled in the published experiments.

However, the delay in induced mutations lasts at least three times as long as the two or three generations of phenotypic lag. The discrepancy may well be due to segregation since cells of *E. coli* are typically multinucleate (80). Phage sensitivity is dominant to resistance in heterozygotes, so that one would anticipate that resistance mutations can come to expression only when there are no sensitive nuclei in the same cell, after enough cell divisions to segregate the mutant nuclei. In *Neurospora tetrasperma*, radiations have a haploidizing effect on heterokaryotic ascospores, and possibly in bacteria (see 20, 52), although the first order kinetics of sterilization of *E. coli* seems to preclude a dominant role for such an effect (102).

Phage resistance mutations have been used to test a variety of chemical compounds for mutagenic activity. Substituted *bis*-(2-chloroethyl)-amines, nitrogen mustards, are outstanding in this respect, and closely simulate the action of radiations both in higher organisms (3) and in microorganisms (14, 17, 93). Of the other compounds tested, sodium desoxycholate has been scrutinized most closely (101). Eight hours exposure of *E. coli* B to a 5 per cent solution at pH 7.7 had a mutafacient and lethal effect equivalent to 100,000 r of x-radiation. The induced mutations were measured only as zero-point mutations, but in view of the long duration of the treatments, the conditions are hardly comparable to those in which radiation was used. Very thorough measures were taken to control possible errors based upon selective survival of mutants as against wild-type cells during the treatment, especially by reconstruction experiments using artificial mixtures of sensitive and resistant cells. However, it would be very difficult to devise controls for a simulation of mutagenic activity by the disclosure of mutations which had occurred previously, but which were camouflaged either by phenotypic lag or by genetic dominance.

From the *E. coli* evidence alone, it could not be demonstrated quite conclusively that sodium desoxycholate is mutagenic, but the report that this compound has a similar effect in *Drosophila* may be taken as a confirmation (101). The mutagenicity of desoxycholate in *E. coli* has been confirmed by Latarjet (48), who reports that cholate is likewise effective.

Among other compounds tested, pyronin Y, Styryl 430, 1,2,5,6 dibenzanthracene endo-succinate, alkyl urethans, caffeine and colchicine, and sodium chloride in high concentrations, are reported to be mutagenic while methyl green, methylcholanthrene, and methylcholanthrene photoöxide are inactive (13, 48, 101). The penetration of the latter two materials into the cell was shown by fluorescence.

Biochemical mutations in bacteria.—Mutations affecting biochemical processes are interesting chiefly for physiological studies, because their application to quantitative mutation studies is limited by technical difficulties. However, a number of artifices have been described which facilitate the isolation of biochemical mutants (19, 29, 55). The most efficient of these utilizes the specific bactericidal action of penicillin on cells capable of growth to eliminate wild type cells inoculated in synthetic medium, while saving the mutants (20, 56).

Some of the biochemical problems which have been attacked recently, using mutants, include sensitivity to penicillin in *Salmonella* (78), carbon and nitrogen metabolism in *Azotobacter* (43, 59) [see also (100)], bioluminescence in *Achromobacter fischeri* (67, 34), the metabolism of proline, phenylalanine, tryosine, and their peptides (32, 87, 88), and of maltose in *E. coli* (27).

Hinshelwood & Peacocke have objected to describing these biochemical variants as mutants (77). After ultraviolet treatment, they isolated 48 colonies of *Aerobacter aerogenes* and studied their growth behavior in artificial media. In some of the tests, a prolongation of lag was observed, but this disappeared after subculture on a complete medium. From the behavior of these cultures, they conclude that biochemical mutants represent merely temporary alterations in the enzyme balance of the cells, reparable by a direct adaptation or training process. It will be agreed that whatever Hinshelwood's cultures were, they probably were not mutants. It is not surprising that no mutants were isolated in these experiments in view of (*a*) the limited number of colonies that were tested and

(*b*) the absence of a period of proliferation after the treatment to take account of the delayed effect.

The present writer has occasionally isolated cultures of *E. coli* like Hinshelwood's, which might be called transitory mutants (see 55, 56), and they have also been seen in *Neurospora*. Hinshelwood's interpretations may well account for some of these, but reverse mutation must also be considered. However, these ill-defined isolates should not be confused with the clear-cut mutants with reproducible, specific, nutritional requirements that are used in genetic and biochemical research.

In contrast to the difficulties of estimating rates of mutation to biochemical deficiency, reverse mutations are rather easily determined, by inoculating washed suspensions into synthetic media, and counting the colonies which develop.

Many biochemical mutants in *E. coli* exhibit spontaneous reversion rates ranging from about 10^{-9} to 10^{-6} per fission cycle. However, reverse-mutations of nutritional requirements in bacteria have not yet been studied genetically to prove that the phenotypic reversion is based on reverse rather than suppressor mutation.

Ryan & Schneider have made detailed measurements of reversion rates in several coli mutants, especially one requiring histidine (82, 83). The adaption of this histidineless mutant to growth on minimal medium is complicated by a selective interaction which depends on the histidine concentration. At minimal levels of histidine the growth of histidineless cells is impaired, while wild-type histidine-independent cells grow freely. At optimal levels, both types of cells proliferate freely, and mixed cultures do not change their composition during growth. At intermediate levels, however, growth of the wild type cells is suppressed, and in mixed cultures with histidineless, the proportion of histidine-independent cells may remain nearly constant. At the time of writing, the series of publications recounting this interesting phenomenon had not been completed. However, it appears likely that the selective phenomena brought to light by the reconstruction experiments account fully for the course of events when the histidineless mutant is inoculated into synthetic media with various levels of histidine. With no histidine, some of the cultures may eventually adapt, and reach a final level of growth equal to that of the wild type in the same medium. The adapted cultures contain a preponderance of cells which are thereafter independent of histidine, and are presumably

the result of selection of a small proportion of spontaneous reversions from histidineless. When intermediate levels of histidine are used, the adaptation is suppressed, and in all of the cultures, the ultimate density is proportional to the amount of histidine added. It is to be supposed that the limiting level of histidine influences the course of selection rather than the rate of the mutation which underlies the adaptation. With optimal levels of histidine, of course, there is no selective pressure to favor the reverse-mutants, and these accumulate only to a negligible extent under mutation pressure. Although the biochemical basis of these selective interactions has not yet been revealed, a parallel example in *Neurospora*, with a more thorough genetic analysis, has been discussed (81).

Guthrie has presented preliminary data on the reversion of a purine-less coli which he interpreted as a direct effect of the medium on the reverse mutation rate (36). However, he now inclines to an interpretation based on modification of selection dynamics, very similar to Ryan's conclusions (37). This type of selective interaction probably accounts also for other examples of an environmental effect on genetic adaptation, e.g., the reversion of the requirement for tryptophane in lactobacilli (104).

The training of typhoid bacteria to dispense with tryptophane is best understood as an example of reverse-mutation (30, 33), although, fortunately, the complications noted with histidineless coli have not been observed.

The term adaptation has always been used rather loosely. To the general biologist, it means only a change in an organism or species which seems to result in a better fit to its local environment. It should not connote the mechanisms by which it is accomplished. In microorganisms, adaptation is often genetic, i.e., is the result of an inherited variation which occurs spontaneously, but which becomes established under the pressure of natural selection just because it results in greater fitness. In Protozoa it seems to be established that an inherited adaptation may sometimes be the result of a direct reaction with an environmental factor (89). However, there is no convincing evidence, as yet, for such an example in the bacteria, except for induced lysogenicity. There are, of course, many examples of direct adaptation, viz., enzymatic adaptation to substrates (where it occurs in nongrowing cultures and natural selection can be excluded), or to changes in salt concentration. However, such adaptations are generally not inherited

and quickly disappear upon the removal of the inciting agent. Direct heritable adaptations may be a reflection of cytoplasmic heredity, a subject of very great interest in genetics today, which is all the more reason why possible examples should be scrutinized most thoroughly, especially to disqualify natural selection.

The biochemical changes which are subsumed under nutritional mutations are, unfortunately, very difficult to analyze enzymatically, although an encouraging start has been made in *Neurospora* with systems involved in the synthesis of tryptophane and of pantothenic acid (71, 97). For studies on gene action in bacteria, effects on the enzymes involved in carbon metabolism should be more easily investigated by the biochemist.

Many descriptions of fermentative variations appear in the literature, dating from Massini's classical description of *E. coli mutabile*. They are readily produced under the influence of mutagens, and easily detected with the help of indicator media such as Levine's Eosin-Methylene Blue Agar (57), or with triphenyl tetrazolium (53). When 10^8 cells of wild type *E. coli* are spread on an indicator agar plate, and exposed to ultraviolet light long enough to reduce the survivors to about 200 to 400 colonies, a yield, e.g., of lactose negative mutants of 1 to 1,000 to 1 to 5,000 is found by inspection. Mutants discovered in this way are, as often as not, in the form of sectors rather than intact mutant colonies. The sectored colonies consist of mutant and nonmutant components, and it seems reasonable to ascribe them to the segregation from a bi- or multinucleate cell, in only one nucleus of which a mutation had occurred. Barring only the contingency that the sectored colonies arose from two cells close together which happened to survive the heavy dose of radiation, they afford direct evidence of such a segregation process, which is, after all, only to be expected from the cytological evidence, if the desoxyribonucleic acid containing bodies in the cell are accepted as nuclei.

Kristensen's studies of fermentative variation in *Salmonella* (47) have shown (*a*) that mutations leading to the ability to ferment a given sugar are spontaneous, and merely selected for by the presence of the sugar, and (*b*) that mutations concerning different sugars are usually independent of each other. However, in *S. typhosa* Type II, one form is inhibited by xylose and ferments dulcitol; the alternative is not inhibited by xylose and fails to ferment dulcitol. He has been able to demonstrate mutation from

one to the other by using xylose and dulcitol media respectively. Unfortunately, this system is not well adapted to accurate measurements of mutation rates in the two directions.

Monod (72, 73) has isolated lactase from wild type *E. coli* and has demonstrated that it is a simple hydrolytic enzyme, strictly adaptive, and absent in mutants which are unable to ferment lactose. He has also compared bacteria whose utilization of other sugars had been intensified by selective culture and found no differences in their capacity to oxidize lactose. He concluded that the formation of lactase was under unitary genic control, i.e., that the enzyme was produced under the influence of one and only one gene. This useful working hypothesis has had wide circulation as the one-to-one theory (8). However, the present writer's experiments have not been in such good accord with it (51). Among several hundred lactose negative mutants at least seven distinct classes have been identified which differ by mutations of different genes, as determined by recombination tests. Two of these classes have alterations in enzymes other than lactase, both being unable to ferment maltose; one glucose negative (27), the other gluconate negative. Since the specificity of adaptation shows that lactase must be distinct from these enzymes, these mutations are pleiotropic, i.e., they influence several enzymes. For these adaptive enzymes, it seems likely that there is not a one-to-one relationship between any gene and the enzyme finally produced, but that the gene impinges on the complex adaptation mechanism. Some of the mutants will produce lactase under conditions of altered temperature or substrate, which is hardly consistent with the hypothesis that the alteration of the gene means the absence of the specificity of the enzyme. For the possibly more direct synthesis of constitutive and biosynthetic enzymes, a simpler relationship between gene and enzyme may hold.

Fermentative mutants, whose stability can be assayed by inspection of colonies, are favorable material for the study of the genetic control of genetic stability (49). Some differences in the reverse-mutability of various lactose negative mutants in *E. coli* are due to allelomorphs of different stability. Both spontaneously, and under the influence of ultraviolet light, derived mutants with lower reversion rates were found, but increased mutability was not. The apparent stability of certain derived stable lines depends on the accretion of a second mutation interfering with the utilization

of lactose. In such a double mutant, the expression of a reversion of either mutant gene is prevented by the other.

Mutations affecting colony color, although having an obscure biochemical basis, are convenient for certain types of mutation and population studies. With such variants in *Phytomonas stewartii*, Lincoln (58) has evaluated the role of mutation and selection in the effects of temperature on the variation of bacterial populations. Temperature has only a relatively small influence on mutation rate, two- or three-fold increases for a rise of 10°C., as in higher organisms, compared to its profound effects on the selective advantages of various types in mixed cultures. Since, according to the most popular theory, spontaneous mutations are thermal accidents, quantitative studies on the effect of temperature changes and temperature shocks on mutation rates assume considerable importance.

Drug resistance mutations.—Mutations conferring resistance to antibacterial agents are of special interest to medical bacteriologists. That such mutations may be induced by contact with the drug is still widely believed, but there is no convincing evidence to substantiate it. Statistical analyses of penicillin (60), sulfonamide (75), and streptomycin (22) resistance in staphylococci and in *E. coli*, along the lines discussed for phage resistance in *E. coli*, support the concept of spontaneous mutation and selection.

Drug resistance is often relative or quantitative rather than sharply qualitative. Firstly, resistance refers to a definite concentration of the drug, and there is often a very sharp cut-off in the proportion of surviving cells with relatively small increases in drug concentration. Secondly, resistance may be variably expressed in a population, to be described as a distribution rather than a single parameter. The distributions of adjacent steps of resistance may overlap. Therefore, experiments on these mutations must be closely controlled to insure that effects on the expression of resistance are nullified.

The metabolism of resistant mutants may be altered, but enzymatic changes are not necessarily the genetic cause of the resistance. But a number of workers have interpreted resistance as the direct injury of cellular enzymes by the drug (18, 84). Since these metabolic changes persist on cultivation in drug-free medium, it would have to be argued (40) that the susceptible enzymes are

autocatalytic, i.e., that the alteration is transmitted in a heritable fashion. This conclusion needs more convincing evidence than is now at hand to substantiate it over the gene theory of inheritance. Genetic resistance is, of course, probably mediated by effects on enzymes, but this conclusion should not be confused with the hypothesis of direct injury (see 61). Because the enzymatic mechanisms of antibacterial action are still largely inaccessible, there has been relatively little work to show different responses of enzymes of resistant mutants to the antibacterial agent. Streptomycin has been reported to inhibit benzoic acid oxidation in sensitive mycobacteria, but not in resistant mutants. Further work (31), however, showed that this effect was not on the oxidative enzymes per se, but on their adaptive formation, concerning which as little is known as about growth as a whole. Sevag & Gots have, however, examined dehydrogenase activity in pneumococci and found evidence for the occurrence of altered enzyme proteins in mutants resistant to a variety of inhibitors (85).

A number of bacteria develop a requirement for streptomycin (70, 76) concomitantly with resistance. Animals injected with streptomycin-dependent meningococci will survive unless they are treated with streptomycin. The biochemical basis of this requirement is as obscure as the mode of action of streptomycin, but is perhaps clarified by experiments on sulfonamide requiring *Neurospora* (108). In this fungus, sulfonamide resistance is sometimes associated with dependence on sulfonamide. If, however, an additional mutation is introduced which prevents the synthesis of *p*-aminobenzoic acid (PAB), sulfonamide is not required unless PAB is added in too large amounts. Apparently, resistance to sulfonamides was effected by a very efficient utilization of PAB, and a sensitivity to excess of it. Normal synthesis of PAB exceeds the sensitivity threshold, so that growth is inhibited unless the PAB antagonist, the sulfonamide, is also added. In streptomycin dependence we may likewise imagine that the resistance mutation is accompanied by an expansion of the sensitive enzyme systems so that they are in balance in the presence of the inhibitor; when uninhibited, their exaggerated activity may be supposed to interfere with normal growth.

Genetic resistance may make growth insensitive to an inhibitor, but leave other processes liable to interference. For example,

mucoid *Brucella abortus* yields mutants whose growth is not affected by streptomycin, but which have a rough rather than a mucoid appearance in its presence (41).

Resistance mutations in staphylococci have been used by Stone, Wyss *et al.* (90, 91, 106, 107) to test for indirect mutagenic effects of radiation. They find that nutrient broth, heavily irradiated with ultraviolet light, induces penicillin resistant mutations. Possible inaccuracies due to selection of pre-existing mutants have been controlled experimentally. Irradiated broth also increased the rate of mutation both to mannitol negative from the normal mannitol positive, and the reversion back to positive. The effects of radiation on broth can be duplicated with hydrogen peroxide, and both treatments can be negated with catalase. However, peroxide applied directly to washed cells has no mutagenic action, and it was therefore concluded that peroxide reacts with some component of the broth to form the mutagenic compound. These experiments pose some interesting problems for the direct effects of radiation. However, they do not, as yet, provide unequivocal support for the hypothesis that "modified substrate molecules may be assimilated by the organism and built into inexact replications of the genetic mechanism." This hypothesis is to be compared to the conception that mutagens act in some way to increase the nonspecific activation energy for chemical change of the gene. The latter notion, long current for radiations, should be extended to nitrogen mustard, because this compound induces reverse-mutations as well as mutations (35). But irradiated broth also increases the rate of reverse-mutation, if, as these authors suggest, the mutation from mannitol negative to positive is to be so regarded. It is difficult to see how a second inexactitude in the replication of a gene, in the specific way envisaged above, could reverse the effect of a first. At any rate, it should be possible to test the hypothesis of analogue assimilation into the gene by thorough comparisons of mutation and reverse mutation rates under the influence of these mutagens, preferably in several diverse systems.

Many studies of direct adaptive resistance allow a prima facie case for natural selection which is at least as good as for the direct response. However, Strandskov (92) has reported some observations on resistance of *E. coli* to 2-chloro-PAB (CPAB), which need a more thorough analysis. The most critical experiment involved the plating of a small number of sensitive cells on CPAB-agar.

After four days, 10 to 25 per cent of the cells developed into colonies which consisted of resistant cells. Since there was clearly no such high proportion of resistants in the original population, it could be inferred that the resistance was induced by the CPAB. It was recorded that resistance was retained through one year of culturing on nutrient agar, i.e,. that it was transmissible. However, it has since been observed (unpublished work of the writer) that the resistant colonies do not develop directly from single cells, but that microcolonies of some thousands of cells form initially in the inhibitor medium. These microcolonies, invisible to the naked eye, open the door to natural selection by providing populations in which spontaneous mutations can occur. It should be emphasized that this observation does not prove natural selection, nor does it disprove direct adaptation, but it does leave the question open for further study.

Resistance to physical agents.—Mutations augmenting bacterial resistance to physical agents have also been found. In *E. coli* B, a mutant, B/*r*, with increased resistance to x-rays and ultraviolet light has been found among the survivors of heavy doses of such radiation (102). Since the mechanism of radiation killing is still under discussion, there is no clear interpretation of the resistance noted in B/*r*. According to Demerec & Latarjet (24), the mutational response to irradiation is the same in B and B/*r* for a given physical dose. The changed response to x-rays is in the form of a flattened slope of the log survivor/dose curves, while with ultraviolet the linear relationship, seen on B, changes to a sigmoid response on B/*r*, indicating that several cumulative hits are required to kill a B/*r* cell. However, the lethal response of B, both to x-rays and ultraviolet, breaks sharply toward resistance at about 1 per cent survival. This break cannot be accounted for in terms of resistant mutants (17), but is, on the other hand, inconsistent with the single-hit theory of radiation sterilization.

In a very recent report, Kelner (45) has indicated that the bactericidal effects of ultraviolet light can be substantially reversed with visible light. Photoreactivation occurs in actinomycete spores, bacteria, and bacteriophage, so that this observation may lead to a significant extension of our understanding of radiobiological effects.

Breaks in the lethal responses of bacteria to sterilization with heat have been frequently noted, but only rarely has the question

of the heritable resistance of the survivors been considered. In *E. coli*, the resistant tail of the distribution of responses to heat does not give rise to a more heat resistant culture when grown out, and the prolonged survival of these cells must be presumed to originate in their physiological condition at the time of treatment rather than in genetic differences (42). However, spores of *B. subtilis* which survived thermal inactivation produced progeny with augmented resistance to heat (21). These observations recall the tolerance to heat which Kluyver & Baars succeeded in developing in sulfate reducers by gradual acclimatization (46). Although they supposed it to be a physiological adaptation, the evidence does not exclude mutation and selection.

The response of *E. coli* to osmotic pressure changes may involve both physiological adaptation and genetic changes (26). By increasing the salt concentration of the medium gradually, most of the cells in a resting suspension can be acclimatized to high salt concentrations; on the other hand, the survivors of sudden changes in concentration give tolerant progeny.

Antigenic variation.—Space does not permit of adequate consideration of the extensive research on *Salmonella* antigenic variation which fortunately, however, has been reviewed recently by Harrison (39). Since then, Bruner & Edwards (10) have demonstrated, with the help of antiserums, changes from the nonspecific 1, 2, 3 . . . phases of a number of types to a 1, 2 . . . phase characteristic of other named types. Therefore, they (11) and Kauffmann (44) have recommended that the 3 antigen be dropped from the diagnostic schema, and that the types which were formerly distinguished by it be coalesced with the 1, 2 . . . named types. In another report (12), they demonstrated induced changes in the somatic antigens. In the presence of X, XXVI antiserum, the homologous component in *S. anatum* and in *S. meleagridis* was replaced by the XV antigen, yielding forms indistinguishable from *S. newington* and *S. cambridge* respectively.

The role of antiserum in these experiments has not been settled definitely, but no compelling reasons have been offered to regard it as other than selective for spontaneously occurring variations.

Serological work on these organisms has proceeded so much further than our understanding of the genetic mechanism involved that it would be fruitless to offer any ready-made explanations. The dimorphic phase variation of flagellar antigens is especially

perplexing, but it may be useful to look for an interpretation in the light of recent studies of the cytoplasmic determination of antigens in *Paramecium* (89).

Braun (9) has reviewed his studies of dissociation in *Brucella*. This work has provided strong support for the role of spontaneous mutation and selection in determining the nature and extent of population changes. Intrinsic differences in the dissociation index were shown to be explicable in terms of growth responses of the original smooth strains, and their various mutants, as could the influence of such environmental factors as temperature, pH, renewal of the medium, and a serum factor. However, it is not clear whether the failure of certain strains to produce the so-called S^r type is due to intrinsic differences in the range of genetic variability, or to selective factors which operate after variations have occurred.

Not all recent students agree that apparent effects of environment on rates of dissociation are due to selective interactions acting after the spontaneous appearance of the mutant. In *Brucella bronchiseptica*, Dickinson noted (25) that variation of a culture from N, normal, to V, a relatively avirulent variant, was suppressed either by a deficiency for chlorides, or by the addition of maleic acid as a carbon source in the presence of adequate chloride. In an attempt to reconstruct the populational relationships, the effect of maleate on the composition of mixed N-V cultures was examined. "In Koser's medium even the weakest mixture became pure V within three or four subcultures," showing that there is a strong selective advantage here. "In chloride free medium mixtures containing up to one loopful of V did not reveal V colonies after twenty subcultures but mixtures containing two loopfuls or more of V became pure V. . . . " This statement shows that chloride strongly influences the selective advantages of V. But the conclusion that maleate affects the mutational process per se, and not selection, cannot be inferred from "In maleate medium, with and without chloride, the weakest mixture tested contained one loopful of V and this rapidly became pure V," since it had been established that "one loopful of V" is a critical density of cells, above which V will predominate in chloride-free medium, and below which it will not. Before conclusions as to the behavior of newly produced variants may be drawn from reconstruction experiments, such experiments must be performed under conditions approxi-

mating as closely as possible those of the variation experiment. This work is quoted at such length because it is a remarkably good example of a difference in the behavior of a population at different initial concentrations. Although it is not established that these salts influence variation per se, it is no less interesting that such materials can so profoundly influence the behavior of a population.

A report (68) whose evaluation is less certain now concerns the influence of acetate on mucoid to smooth variation in a group C hemolytic streptococcus. This organism is unstable in medium lacking acetate, and cultures rapidly become smooth after three to six transfers, although the mucoid form is perpetuated in the presence of acetate. The smooth variants are stable, not reverting to mucoid during 14 transfers in acetate medium, so that we have a heritable variation, not an environmental effect. In order to dispose of selection "two single S phase cells were mixed in acetate-containing broth containing 5,000,000 M phase cells. After eighteen hours incubation at 37°C. equal numbers of M and S forms were present." The authors therefore conclude that whether acetate is present or not, the S form has such a powerful selective advantage that, if formed, it should predominate. This is certainly the kind of evidence which should be adduced in support of their hypothesis, but it is regrettable that a more complete study of the population dynamics has not yet been made. It may be pointed out that the S phase consists of long chains of cocci, so that many more than two cells were undoubtedly introduced in this limited reconstruction experiment. It is interesting to notice that S has such a selective advantage in view of the remark that "the growth rates of (S and M) in both . . . media were identical. . . . " Such interactions not explainable in terms of the growth rates of the isolated cultures are quite common. However, these combinations can be expected to be the most unstable, especially with respect to the proportion of cells of the variant which is initially needed for it to predominate. If further work confirms this response as induced directly by the absence of acetate, it would probably be best explained as depletion of a cytoplasmic factor, like the attenuation of kappa in *Paramecium* (79). Kinetic studies on the rate of transformation of individual cells and their progeny in acetate free medium would then give considerable insight into the mechanism. However, it still seems an open question to this reviewer whether these results are best accounted for by direct induction or by spontaneous mutation and selection.

It would seem that there are no well substantiated examples of direct adaptation in bacteria. However, the gradual attenuation of virulence in *Phytomonas tumefaciens* cultured on glycine, resulting ultimately in an irreversible loss of pathogenicity, may be a promising example of the quantitative modification of a cytoplasmic genetic factor (95).

"Infective Transmission": Type Transformation and Induced Lysogenicity

The story of type transformations in pneumococci must be familiar by now to all readers of these reviews. Since last surveyed (66) there has been only one report dealing with this system. MacLeod & Krauss (64) have found an intermediate Type II, which is visibly acapsulate, but which produces serologically detectable specific polysaccharide. Competent rough strains are transformed to this intermediate smooth under the influence of polymeric desoxyribonucleic acid extracted from it. The intermediates were susceptible to further transformation to the typical smooth Type II, but unlike the roughs from which they were derived, could not be transformed into other smooth strains. In addition, the intermediate to smooth II transformation was not brought about by transforming principles isolated from other pneumococcal types. Evidently we are not dealing with a simple "intensifier" factor. It is not yet known whether transforming factors operate in pneumococci for characters other than the capsular polysaccharide. It may be recalled that the host specificity for rabbits of a pneumococcus strain was invariant under capsular transformation, suggesting a "somatic" basis for this character, while pathogenicity for mice in other types was correlated with transformation to and from type XIV polysaccharide, and is apparently connected directly with it (65, 86). No further evidence has been brought to bear on the question, raised primarily by Mirsky, as to the sufficiency of the desoxyribonucleic acid component of the transforming principles, and whether protein contaminant is responsible for its specificity. In this connection, a detailed publication of the data mentioned by Avery *et al.* (4) on the electrophoretic and ultracentrifugal homogeneity of the active preparations, and the size and shape determinations would be desirable.

There have been several further reports of transformations in other bacteria, without enough details to permit of an analysis. Wyss (105) and Voureka (96) have reported that staphylococci

and *E. coli* grown in the presence of extracts of drug resistant bacteria acquired a heritable resistance to the drugs concerned. However, there might have been a physiological potentiation of resistance followed by spontaneous mutation and selection in the populations thus permitted to develop. In analogy to the pneumococcus transformation, a conversion of a nonmotile *Bacillus mesentericus* to a motile *B. anthracis* is reported (69).

Our present information on transformations does not allow any reliable judgments on their bearing on the genetic processes observed more usually in higher forms. The more credible reports uniformly picture the acquisition of a genetic function, and from purely mechanical considerations it would seem most likely that the transforming agents are incorporated into a cytoplasmic system like that of kappa to perform such functions. There would also seem to be a parallelism with the phenomenon of induced lysogenicity, which has been pointed out before, but which deserves greater emphasis, especially in view of the apparent justification of Altenburg's "viroid" theory of kappa (1).

Although most encounters between a phage particle and a "sensitive" bacterium result in the destruction of the cell, certain combinations may result in the establishment of a symbiotic relationship, whereby the phage multiplies without apparent injury to the bacterium, and on the other hand, the bacterium may acquire resistance to other particles of the same or other phages, presumably by the "interference effect" (15). Very little is known of the critical conditions which determine whether lysogenicity or lysis will be the issue, except that in *Bacillus*, lysogenicity may be related to the adsorption of phage just prior to sporulation of the bacteria. Once established, it appears to be very difficult to disinfect the phage, as Burnet states, "The permanence of the lysogenic character makes it necessary to assume the presence of bacteriophage or its anlage in every cell of the culture, i.e., it is part of the hereditary constitution of the strain" (16). Now, with respect to resistance to other phages, a lysogenic bacterium has a cytoplasmic genetic determination of this character, and one, moreover, which is capable of transformation by the use of extracts, i.e., preparations of the lysogenic phage. This is shown especially well in studies on phage typing of staphylococci, in which the primary determination of resistance patterns seems to depend on lysogenicity, genetic differences playing only a secondary role (99).

Many organisms are lysogenic as first revealed when they are tested on appropriate sensitive indicator strains (16). Since a limited number of indicator strains are used, it is quite possible that most bacteria carry these symbionts. The activity of these agents may well account for the transformations of virulence observed in *S. typhi-murium* (63) and possibly, if lysogenicity affects antigenic qualities, the transformations observed in *E. coli* (7) and in *Shigella* (98).

If the lytic activity of the symbionts were not apparent owing to the lack of suitable indicator strains, the incidental effects would have to be regarded as determined by a cytoplasmic factor. Furthermore, since the symbiotic phage is subject to mutation, and the bacterium-phage system to natural selection, one can speculate that functions ordinarily relegated to the nuclear mechanisms might be taken over by the symbionts, along the lines suggested by Altenburg and Darlington. But for this type of evolution in bacteria, proof is still wanting.

Genetic Recombination

The question of a sexual phase in bacteria has been moot for many years, but genetic evidence to support it is now available for *Escherichia coli* K-12. Suggested by the occurrence of prototrophic recombinants in mixtures of complementary biochemical mutants (94) the hypothesis of sexuality was strengthened by a study (50) of the segregation of other characters, including sugar fermentations and phage resistance, in a way that indicated linear linkages. Some of these findings have been retested and confirmed (38). Finally, stocks have been described (54) in which a diploid heterozygote can be isolated, and allowed to segregate, yielding various recombination classes, as well as the character combinations of parents. The discovery of these heterozygotes would seem to make very remote any interpretation of recombination based upon transforming factors like those described in pneumococci.

It cannot be said how important recombination is in the genetic variation of other bacteria, for studies are still in progress to determine its occurrence elsewhere than in K-12. However, recombination can only reshuffle mutations which have already occurred, and is not a primary source of variation. Therefore, perhaps the most important aspect of genetic recombination to students of variation is as a tool for their analysis of bacterial variation.

Cytological evidence for processes of nuclear fusion is still controversial [see (80)]. It has not yet been possible to correlate cytological with genetic studies on any one organism, but with the material now available, we may look forward to the establishment of a bacterial cytogenetics.

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THE MORPHOLOGY, CYTOLOGY, AND TAXONOMY OF THE ACTINOMYCETES

BY DAGNY ERIKSON

Research Officer, Agricultural Research Council, attached to Rothamsted Experimental Station, Harpenden, Herts, England

INTRODUCTION

In the bare decade that has elapsed between the publication of the Fifth (1) and Sixth (2) Editions of *Bergey's Manual of Determinative Bacteriology*, there has been an unprecedented rise in actinomycete stock. Even the most casual reading of the current journals reveals that in the present-day assessment of Man-Microbe values actinomycetes are of almost gilt-edged importance. It would be gratifying to report that the incomparably greater attention now paid to these microorganisms is due to the intrinsic fascination of the *Actinomycetales* as a group that is unique in the whole system of microbiology; that bacteriologists and mycologists have laboured together to solve once and for all the question—are they minute fungi, higher bacteria, ancestral prototypes of both, or intermediates; that medical and plant pathologists have experimented in common to discover whether there is any similarity in the mechanism of infection brought about by the agents of such diverse diseases as lumpy jaw of cattle, Madura foot of man, and scab of potato; that agronomists have accounted satisfactorily for their ubiquity in soil, sand, snow, and Bikini atoll (3); that systematists have been impelled to bring textbook order out of natural chaos by the intellectual joy of grappling with organisms whose extraordinary morphological plasticity is evident in the 23 different generic names which they have borne at one time or another. This would indeed be a pleasing picture, and perhaps it may confront the reviewer of a decade hence. For the present, and for credibility's sake, it is only necessary to remind the reader that the recent increase in the volume of work done on the actinomycetes has coincided with the discovery of streptothricin, streptomycin, and similar antibiotic agents by Waksman and co-workers (4).

Nevertheless, the academic student has reason to be grateful

for the assiduity of his practical fellows. A great advance has been made in the understanding of these organisms. Reading the eighty-eight pages of the 1948 Edition of *Bergey's Manual* devoted to the *Actinomycetales*, it is no longer possible to complain with Jensen (5) that "there is an ever increasing tendency to make the Order *Actinomycetales* what the *Fungi imperfecti* are among the *Eumycetes*—a heterogeneous collection of forms which cannot conveniently be placed elsewhere in the system." A rational approach to the problem of classification has been made by Waksman & Henrici (6), which stems from the fundamental work of Ørskov (7). The desirability of the particular names selected will be considered in the final discussion on Taxonomy. Throughout the main sections on Morphology and Cytology, the material will be discussed in the order, and using the nomenclature, given by *Bergey's Manual*.

FAMILY: ACTINOMYCETACEAE

Genus: Nocardia

Vegetative mycelium.—The chief morphological criterion for actinomycetes in general is that they produce filamentous growth with true branching. Filaments can be produced in abundance by several of the eubacteria, for instance, the influenza bacillus and aerobic spore-formers, when growing rapidly on moist media. Branching itself is defined in *Zinsser's Textbook of Bacteriology* (8) as a specialized form of budding and has been noted for more than 70 different species of bacteria. There are innumerable pictures of Y-forms or "3-point multiplication" in the literature for both coryne- and mycobacteria. To discriminate between the occasional more profusely branched filaments of certain mycobacteria and the very similar, short-lived, minute mycelia of many nocardias [*Proactinomyces* Jensen (5)] is often impossible in individual cases. Jensen (9) has emphasised that the essential difference is the consistent development of a mycelium in the initial stages of nocardial growth. McCarter & Hastings (10) have pointed out the frequency with which very closely apposed cells, which have recently undergone angular division, can at lower magnifications readily appear as branched forms. The just criticism has also been made by Umbreit (11) that there is no difference in kind, only one of degree, between a mycobacterial filament with one or two branches and a nocardial mycelium showing five to seven branches. This gradual transition from one group to the other was clearly demonstrated in the origi-

nal studies of Ørskov (7) and must be accepted as another of the innumerable instances in which Nature prodigally overlaps man-made *taxonomic boundaries*.

Accepting the initial production of a branched mycelium, of whatever size, as the characteristic feature of all actinomycetes, the genus *Nocardia* (Ørskov's Groups IIa and IIb) is peculiar in the more or less rapid segmentation of that mycelium into irregularly shaped cells, filaments, rods, or cocci. It is this marked polymorphism which has been responsible for much confusion in the descriptions of many of these forms. The oldest known organisms of this group are pathogens, e.g., *N. farcinica* and *N. asteroides* and it is in the medical literature that we first find accounts of "typical bacilliform nocardial hyphae with rounded bodies or spores" [Chalmers & Archibald (12)]. With the gradual discovery of many morphologically similar organisms to be found in soils, a clearer insight has been gained of the various modes in which the initial mycelium may divide.

Multiplication.—Rapid subdivision into diverse cell elements is readily seen in members of the *N. corallina* species-group. First described as *Bacillus mycoides corallinus* by Hefferan (13), the type species was recognised as a nocardia by Jensen (9). His drawings agree with the photographs of Topping (14) for her Group 2b organisms in showing very clearly the segmentation of the branching mycelium into short rods within one day, and into cocci in two to five days. Krassilnikov (15), following the development of similar types in hanging drop cultures, noted that a new sprout is formed at the end of a filament approximately every 55 to 75 min., so that after 24 to 48 hr. multiplication is such that every new cell split off becomes shorter and shorter, the cells finally attaining a spherical shape. These spherical cells multiply by budding with the result that the culture soon has the appearance of being composed of micrococci. Novak & Henrici (16), studying an undoubted nocardia of this type, found that the coccoid bodies often multiply in three planes, thus producing a staphylococcoid arrangement. A streptococcoid appearance, as the result of division in one plane, has frequently been observed by Erikson (17).

Concentration and segmentation of the protoplasm within a filamentous cell, followed by dissolution of the enclosing membrane, that is, the production of gonidia, has also been described by Krassilnikov (15). Novak & Henrici (16) also noticed rounding

up of the protoplasm within a filament, and the subsequent disappearance of the portions of filament between the rounded masses, but were unable to determine whether the wall of the filament dissolved or was ruptured, since Brownian movement interfered with observation of such minute forms. Their conclusions are shared by Erikson (17), who found pictures similar to those of Krassilnikov (15) in stained preparations of *N. salmonicolor*, but who was unable to discover convincing evidence of the sporogenous nature of these granules in the living material. "Chlamydospores" and "resting spores" of different shapes have been seen in old cultures by Grigoraki (18) and other writers, but, as Baldacci (19) points out, in the experience of most workers these can be ascribed to the degeneration of the filaments. No well authenticated instances of "spores" consistently occurring in the vegetative mycelium under a variety of cultural conditions have yet been reported. The "cystites" of Jensen (9) and the "involution forms" of Ørskov (20) seem vegetative in origin.

The making of pronouncements on the basis of post-mortem material is an unfortunate legacy of the early days of medical bacteriology. It is only too easy, as every student of actinomycetes knows, to make stained preparations from different cultures of the same strain, and to find here and there peculiar condensations of the protoplasm within certain filaments or fragmented cells. No one can question the objective existence of such structures. Their interpretation is another matter. Fragmented portions of the vegetative mycelium will usually develop into a fresh mycelium in a new environment, sometimes by lateral budding; sometimes by elongation in one or two, or even three planes; that is, by germ tubes. The morphologist who agrees with Henrici (21) that "comparative morphology and taxonomy will ever be the firm foundation upon which a knowledge of biological science, pure or applied, must be built," attempts to interpret his findings in the light of physiology, and to refrain as far as possible from the mortician's outlook. All the workers mentioned above, who have steadily watched the living material, agree that the influence of environmental conditions is one of the most potent factors in determining the course of development and the predominant shape of the cells. Thus, both Jensen (9) and Novak & Henrici (16) noted that glucose-containing media frequently favoured the production of cocci. Erikson (17) found that with a number of these organisms

the production of long filaments showing mainly angular branching into rods with a typical rhizoid appearance was characteristic of growth on solid starch substrates, while the mycelial form with very little segmentation might persist for a week or more on ammonium acetate agar. Again, exceptionally long and persistent filamentous forms often appear in milk.

The influence of population pressure as multiplication proceeds rapidly within a restricted volume of liquid, as in a hanging drop culture, is clearly an operative factor in determining the length of the newly formed cells. This can be deduced from the fact that it is frequently possible to see much longer filaments extending along the cover slip, where the margins of the droplet have spread in a thin film and growth is only one cell deep. In the same way one finds the well-known arborescent projections composed mainly of long and medium-sized cells, which are formed on solid media as certain nocardial colonies age. However, similar phenomena, although without true branching, may be seen in various eubacteria. It is interesting to note that Gordon (22) was able to bring about loss of the characteristic rhizoid mode of growth of *Bacillus mycoides* by culturing the organism in large volumes of broth. In this connection one may also bear in mind the recent discoveries that penicillin and similar substances can inhibit the division of short rod forms such as *Escherichia coli* so that they continue to extend in the long axis of growth and so produce filaments. The nocardias of this group with their spontaneous division into short rods and cocci would be a very suitable subject for detailed investigations using substances which promote and inhibit cell division.

Some accurate information on the nature and permeability of the cell membrances would also be of help in making a just appraisal of the advantages and disadvantages of filamentous growth, and of the conditions under which it is discontinued. Since no other group of microorganisms exhibits such extraordinary plasticity, it is surely time that modern biophysical and cytochemical methods were employed in an effort to solve this problem. Simply to use the time-honoured phrase "life cycle" is no explanation.

Before passing from this "unstable mycelium" group to those approximating the true actinomycetes, it is necessary to mention that Topping (14) and Ørskov (20) have reported soil nocardias which grow in pin-point colonies and show motility at certain

stages. Their observations have not been confirmed by other workers. The writer recently had the opportunity of examining about 300 nocardial isolates directly obtained from soil, but was unable to discover any truly motile strains amongst them. Topping's pictures (14) of peritrichous flagella attached to branched forms in stained slide cultures are not convincing and should be examined in the light of Pijper's general observations (23) on shape and motility in bacteria.

Aerial mycelium.—Ørskov (7) originally distinguished between Group IIa, with aerial mycelium, and Group IIb, without aerial mycelium. The former type is associated with a more stable, richly branched, vegetative mycelium, and approaches the true actinomycetes (*Streptomyces*) in structure; the latter is the soft, mycobacterial, unstable mycelium type which we have just discussed. Although this distinction is, at least macroscopically, valid to a considerable extent, there are innumerable transitional varieties. As the drawings of Jensen (5, 9) and of Erikson (24) clearly demonstrate, minute aerial filaments of one to ten microns in length can be seen by focussing upwards from the transient mycelial growth of many soft bacterial types such as *N. salmonicolor* and *N. lutea*. These rudimentary, or perhaps vestigial, structures arise by lateral budding, and, when a cover slip is pressed over the growth, they are indistinguishable from the substratum filaments, being neither wider nor more refractile. When stained, they exhibit undifferentiated protoplasm throughout. Their appearance is stimulated by growth on poor media: tap-water agar, Czapek's sucrose- or glucose-nitrate agar, starch media, or ammonium acetate agar. They are sterile and abortive, and usually wither away in two to five days. Yet their existence poses profound and far-reaching questions.

Far more than the occurrence of branching, which can be found in some eubacteria under certain conditions, the outstanding, and literally unique, property of the actinomycetes in general is this growth into the third dimension, away from the nutritive substrate. In the saprophytic soil streptomycetes, where this aerial growth is universal and the individual spore-bearing filaments show constitutional differences, it is easy to understand the advantages conferred by an air-borne mode of sporulation. But in the extreme cases of certain soft nocardias, the biological significance of an aerial sprout or two seems very slight. In the intermediate range,

there are many species, such as *N. paraffinae*, *N. asteroides*, and *N. transvalensis*, which normally produce a good firm vegetative mycelium as well as a moderate and visible quantity of aerial mycelium; and the latter may be as long-lived as the former. But, as all students of the group agree, these simple straight aerial filaments show no differential staining as regards their cell walls or protoplasmic contents. They may be divided by transverse septa into irregular cells, which grow like any other cells in a new environment, but they are not spores in the sense of the definition: "bodies identical to one another in form, having a special mode of formation." Nor have they been shown to possess the augmented powers of resistance to desiccation which characterise streptomycete spores. Apart from their extension upwards into the air, they are in no way different from the component cells of the vegetative substratum mycelium.

Krassilnikov (25) has emphasised the lack of profuse branching or spiral twisting in the aerial filaments of this group. Most workers confirm this. It is well worth remembering that in the asporogenous sectors and colonies that may arise by spontaneous dissociation in streptomycete cultures, Appleby (26) for *S. griseus*, and Erikson (27) for *S. coelicolor* have noted that the scant aerial filaments are of this type: devoid of branches and spirals, and divided into sharp-ended, cylindrical cells. It would seem that loss by degeneration in the more highly organised streptomycete results in forms which are analogous to the nocardial norm. The reverse phenomenon, induced spirals and spore production in the aerial mycelium of a nocardia, has not been reported. Both pathogens and saprophytes in this group show the same characteristics.

Acid-fastness.—Fragmented portions of the mycelium of certain pathogens such as *N. asteroides* and *N. leishmanii* exhibit in histological section acid-fast staining properties which are similar to those of the tubercle bacillus and other mycobacteria. In culture, however, they are generally only partially acid-fast. The discovery that a large class of saprophytic soil nocardias had the same staining reactions has led to a classification by Jensen (9), which is based on this property. Since acid-fastness is also correlated with a marked refractility of the living vegetative cells especially on solid media, and with certain biochemical characters such as the absence of diastatic and proteolytic enzymes together with the ability to utilise paraffin, it appears to have some diagnostic value.

All reports on acid-fastness of nocardias emphasise its partial and inconsistent nature, as well as the potent influence of environmental factors. Not only is a single strain positive in one medium and negative in another, but on the same slide and in the same mycelial fragment some cells are positive and others negative; nor is any particular cell element—filament, rod, or coccus, consistently positive for any one strain under identical conditions. Moreover, not even the most clearly positive cells resist alcoholic acid decolorization for the standard period of the Ziehl-Nielsen technique. As Umbreit (11) suggested, it is advisable to curtail very appreciably the time allowed for decolorization in order to differentiate between acid-fast and nonacid-fast nocardias. After examining a large number of strains directly isolated from soil, Erikson (17) confirmed the findings of Jensen (5, 9) that milk favours the development of acid-fastness at the period of maximum growth of the organism. Additions of liquid or solid paraffin, and of increasing quantities of glycerol, to simple synthetic media also had the same effect. Some of the strains, which Gray & Thornton (28) and Turfitt (29) have shown to be capable of attacking resistant substances like phenols and sterols, exhibited enhanced acid-fastness in media containing *m*-cresol. These facts suggest that the organisms which have an inherent capacity for utilising certain substances may distribute some of the products of these reactions within their cells in such a way as to affect the permeability of their cell membranes with respect to basic dyestuffs. The observations of Yegian & Baisden (30) and Yegian & Vanderlinde (31) on the permeability of mycobacterial cell membranes are of relevance here. It must not be forgotten, however, that with the nocardial mode of growth, branching filaments and chains of interconnected cells, there may easily be other factors operative in the distribution of various substances in the cytoplasmic membranes.

Beading of filaments is very common in many species, but the beads are cyanophilic at an early stage of growth. That is to say, the nocardial cell may exhibit in youth an aspect reminiscent of the aged tubercle bacillus, which Laporte (32) has attributed to progressive disintegration. Here it may be worth recalling the pronounced refractility of the cells of the partially acid-fast nocardias. The two characters seem constantly associated, as Jensen (9) pointed out. Thus, the writer recently re-examined *N. lutea*, which Erikson (24) earlier noted as nonacid-fast on

complex protein media, although the living cells on water agar were described as being so filled with bright granules as to appear banded. When the same strain was grown in Czapek solution plus paraffin or a high concentration of glycerol, it was found to be partially acid-fast.

The differences in top and bottom growth on liquid media such as nutrient glucose broth have been investigated to a slight extent. The softer nocardial varieties tend to grow diffusely at first and then to sediment to the bottom, gradually leaving the liquid clear. Some of the more stable mycelium type produce a considerable surface growth of a harder, somewhat waxy consistency. In some species such as *N. opaca*, Erikson (17) was able to establish a rather higher proportion of acid-fast cells in the surface than in the bottom growth, but in general no clear-cut distinction could be made. Alexander & Soltys (33) have shown that in low surface tension media dispersed and submerged mycobacterial growth is possible. With the great variety of nontoxic substances capable of altering the energy relationships at interfaces that are now available, it might be possible to gain some much needed information on the surface activities of the differently shaped nocardial cells of this group.

In fact, the underlying causes of the very erratic reactions of this group towards acid-fast staining are still to be found. Apart from the work of Gordon & Hagan (34), there has been little attempt to correlate this property in the soil species with pathogenicity for animals. Recently, Drake (35) showed that certain pathogenic acid-fast species, especially strains of *N. asteroides*, were resistant to penicillin. It is perhaps worth noting here, too, that Petrik (36), when investigating the desoxyribonucleic acid content of various atypical acid-fast microorganisms, found some evidence that this content was higher in certain virulent mycobacterial strains than in the avirulent strains.

Genus: Actinomyces

Morphologically, the causative agents of actinomycosis in man and cattle cannot be differentiated from the nonacid-fast nocardias which fragment into rods, seldom into cocci, and develop little or no aerial mycelium. Thus, *A. israeli* is a firm-textured mycelial type with occasional nonsporing aerial hyphae; *A. bovis* is a soft smooth-growing organism without aerial mycelium, in which the

initial vegetative mycelium is quickly divided into diphtheroid rods. It is in their microaerophilic or anaerobic requirements and their specialised nutritive demands that they exhibit the characters of obligate parasites, which set them apart from all other actinomycetes.

A. israeli is the classical type species which many workers (7, 37 to 44) have described in detail. There is a voluminous medical and veterinary literature which, in its earlier contributions to the cytology of the causal organism, was concerned mainly with an attempt to reproduce in culture terminal swellings of the actinomyces filaments and to correlate these with the well-known eosinophilic clubs found in morbid tissues. Wright (38) obtained such structures by growing the organism in high concentrations of serum; Naeslund (40), in high concentrations of ascitic fluid; Bayne-Jones (45), in glucose broth; Erikson (43), in semisolid serum agar containing salicin or lactose. Such findings have been inconstant, and it is now generally recognised, as a result of the work of Magrou (46) and Magnusson (47), that the clubs in tissue represent a mechanism of the host against invasion.

There have also been many exponents of the "life-cycle theory," who, seizing upon the various brightly stained granules, "segmentation spores," the alleged sexual "Vierhyphensporen" of Lieske (48) and of Antonioli (49), have found filtrable forms and a complete set of transitional stages between this anaerobic pathogen and the free-living aerobes of the *Streptomyces* group. The experimental evidence for such cycles was reviewed by Erikson (43), who carried out ultrafiltration studies with a variety of strains of both groups which yielded no support for such claims.

The endogenous origin of actinomycosis is now widely accepted, as a result of the discovery in normal mouths of organisms identical in morphology and growth habits with *A. israeli*. Naeslund (40), Lord & Trevett (50), Emmons (51), and subsequent workers have established the presence of anaerobic actinomycetes in tonsillar crypts and carious teeth. Recently, Rosebury (52) has reviewed the large volume of literature which has now accumulated concerning the occurrence of *A. israeli* in the flora of normal mouths, and its significance with regard to the formation of salivary calculus.

Some of these forms from undiseased mucous membranes are of a softer smoother type, resembling *A. bovis*, which is the species

generally isolated from actinomycosis in animals. Lentze (53) describes such an S type as being obtained by dissociation from the R form, or normal, richly branched, mycelial colonies of *A. israeli*. Erikson (43), however, was unable to find any stable dissociants, or any serological relationships between the human and bovine organisms studied. Yet, as with the nocardias, so with this group too, there evidently exists in nature a considerable range of transitional types. With a more precise knowledge of the factors restricting and promoting mycelial growth and inducing cell division, it may be possible to understand the operation of these factors under parasitic conditions. The differences between R and S colonies in the connotation applied to actinomycetes merely means variation in the rate at which the vegetative mycelium divides. It is interesting to note that Garrod (54) and Roberts, Tubbs & Bates (55) have recently isolated and examined human strains which require carbon dioxide as well as reduced oxygen tension for growth, and which produce viscous growth in liquids and soft colonies on solids. Such varieties have also been recorded by Holm (56), who made the significant observation that whole colonies of typical *israeli* mycelium were more resistant to penicillin than the soft diphtheroid colonies, although suspensions of the two types were equally sensitive.

FAMILY: STREPTOMYCETACEAE

Genus: Streptomyces

This genus comprises the most widely distributed and commonly known aerobic saprophytic actinomycetes of the soil, although it also includes a few plant pathogens, e.g., *S. scabies*, causative organism of potato scab, as well as a limited number of species which have been isolated from animal lesions. It is with *S. lavendulae*, *S. griseus*, *S. antibioticus*, and other such soil species capable of producing antibiotic substances, that most modern investigators are concerned, The remarkable advances in the physiology and biochemistry of these organisms, which have been made by Waksman and co-workers (57, 58, 59), are outside the scope of this article. No attempt will therefore be made to deal even cursorily with the vast literature that is accumulating about these particular species, and only an arbitrary selection will be taken of those reports which have a general bearing on the chief morphological features of the group.

Vegetative mycelium.—The essential difference between the *Streptomycetaceae* and the *Actinomycetaceae* of the preceding section is that established by Ørskov (7): the vegetative mycelium of the former does not segment spontaneously into bacillary or coccoid elements. This mycelium develops homogeneously, according to species and the nature of the substrate, to produce the tough-textured, often cartilaginous growth of the typical actinomycete that is such a common air contaminant on bacterial plates. Jensen (5) states that "the mycelium remains nonseptate and coherent even in very old cultures—months and even years—falling to pieces only as a result of local processes of degeneration in the hyphae, which usually retain their uniform thickness." This is also the view of Ørskov (7), Lieske (48), Erikson (24), Waksman (60), and Plotho (61).

Recently, these widely accepted observations have been challenged by Klieneberger-Nobel (62), who, by means of Robinow's method (63) for the staining of membranes, demonstrated certain structures in young filaments of four streptomyces species, which she interpreted as septa. While it is not disputed that dense, dark-staining, often discoid bodies extending across the width of the hypha can be found at very irregular intervals by the use of the tannic acid mordanting technique, it should be emphasised that no septa can be observed in the living organism with the best optical system, or in stained preparations made without previous mordanting. The streptomycetes which grow naturally across Cholodny slides in soils or on buried cover slips have sometimes been found by Erikson (64) to show segmentation of portions of the vegetative mycelium as the moisture content decreased. The dividing walls in these instances were readily seen in the unstained and stained slides and were of the same thickness and aspect as the outer walls of the filaments. According to the electron microscope studies of Carvajal (116), no true septa were visible in young mycelia of *S. griseus*, but occasionally appeared in older mycelia. The occasional strains reported by Jensen (5), Lachner-Sandoval (65), and Plotho (61) of that rare section of this group (Jensen's Ib, Ørskov's "*Act. Affanassiew*"), that have a similar sporulating aerial mycelium but show segmentation of the vegetative mycelium ("segmentation spores"), also produce dividing walls which, according to the descriptions of these authors, are similar in appearance and properties to the outer cell walls of the mycelium.

The internal septa demonstrated by Klieneberger-Nobel (62) are clearly different in constitution from the outer walls. It should be remembered that tannic acid is a protein precipitant, and, following the use of osmic acid as a fixative, it may render visible in the streptomyces filament structures which are different from the bacterial membranes similarly stained by Robinow (63). Moreover, septation, where it does occur visibly, among the parasitic actinomycetes, in the nocardias, and in the streptomycetes just mentioned, is followed by a ready rupturing of the mycelium. As all students of this group know, the typical healthy streptomyces colony is exceedingly difficult to break up. That is the cause of its well-known property: clarity of growth in liquid media.

Influence of environment on vegetative growth.—In their natural habitat of the soil, all streptomycetes exhibit a uniform mode of growth, a very thin, loose, straggling, colourless vegetative mycelium, that gives rise to a few sporing branches at suitable air spaces. Jensen (66) found that mycelial density was favoured by relatively low moisture content and by increase in temperature between 5° and 28°C. Lutman (67) has contended that streptomycetes occur in soil in the vegetative phase, and that the sporogenous hyphae frequently seen on Cholodny slides are due to the unnatural disturbance of the soil particles and consequent introduction of air spaces. The preponderant weight of the evidence given by various workers (64, 68, 69, 70) inclines to the view that in their natural existence in soil, the vegetative mycelial branches often give rise to sporogenous hyphae in the minute air spaces between the soil crumbs. This is what one might expect, since the air-borne spores are admirably adapted for dissemination through porous soils.

If the vegetative mycelium of all species presents the same simple morphological characters in the soil and on simple substrates like soil extract agar, on artificial cultivation with complex media a bewildering variety of growths can be obtained. In Lieske's monograph (48) there are many beautiful plates of variously coloured, shaped, and textured colonies which one organism may produce on different media. The early work of Waksman & Curtis (70) on nitrogen and carbon assimilation paved the way for a better understanding of these phenomena. In general, increased bulk of vegetative mycelium may be ascribed to the availability of excess nitrogen, usually of a protein nature. An extreme case of

vegetative development is the smooth, hard, raised, lichnoid growth which adheres so strongly to such media as blood and serum agar, many peptone agars, and even synthetic agars to which a phospholipid like lecithin has been added (64). The dense texture is due to the closely packed ramifications of the mycelium. No sufficient explanation has yet been given of the characteristic surface gloss of such colonies, which frequently fail to produce any sporogenous hyphae. When the colony is with difficulty broken up and stained, many portions of the filaments show uneven or negative gram staining. The fragments are often nonviable.

Autolysis of the vegetative growth.—Dmitrieff & Souteeff (71) reported autolysis of a smooth colony variant of *A. bovis* Bostroem (probably *S. albus*), while Stanier (72), working with agar-decomposing strains of *S. coelicolor*, found a type of colony which soon died out owing to its rapid autolysis. Such rapid autolysis is not common in the majority of streptomycetes under ordinary cultural conditions. Dmitrieff (73), working a year or two later with the same strains, was unable to plate out a similar autolysing colony variant. The writer, who had some of Stanier's agar-decomposing *S. coelicolor* strains under continuous observation for nearly two years, also failed to discover such an autolysing colony type.

However, the recent development of the technique of submerged vegetative growth for use in large-scale plants producing streptomycin has brought into prominence the facility with which an abundant vegetative mycelium autolyses under such conditions. Lumb (74) states that with *S. griseus* such a rapid mycelial development takes place within the first 20 to 24 hr. that it may account for as much as 0.6 per cent weight per volume of liquor. This is followed by a second phase which lasts until about the forty-eighth hour of the fermentation, during which time there is widespread autolysis of the mycelium accompanied by the maximum yield of streptomycin. Thus, enhanced vegetative multiplication at the expense of an abundant food supply, under conditions of continuous aeration, sets in motion the processes of autolysis and decay at a very early date.

In stationary liquid cultures the characteristic puffball colonies, which Ørskov (7) has described as gradually filling up the entire contents of tubes of broth, will remain intact and coherent for many months. The minute colonies, which often grow attached

to one side of the wall of the tube (24) in simple synthetic solutions, can be noted in the same positions at least six months later. This is even the case where the medium contains such minimal concentrations of nutrients as 0.01 per cent sodium nitrate and 0.002 per cent glucose (64). As long as the liquid covers the growth, although the nutrients may be exhausted, the mycelium apparently remains in a state of arrested development, showing very few signs of local degeneration for quite prolonged periods. On subculture to fresh media such months-old colonies are generally viable. The superior longevity and greater resistance to autolysis shown by the vegetative growth in stationary liquid cultures of simple composition, as compared with similar growths on solid media, are probably to be ascribed to the readier diffusibility of injurious metabolites in the liquid media and and their consequent dilution. Enforced aeration evidently accelerates metabolism to such an extent that growth is almost contemporaneous with decay.

Goryunova (75), in comparing the processes of autolysis with the activities shown by certain of the many streptomycetes which lyse bacteria, especially gram positive bacteria, states that both the autolysis of the streptomycetes and the lysis which they induce are accompanied by deterioration of proteins; the latter, however, proceeds with a lower proteolytic activity, proceeds further, and at a different pH level. In recent years the bacteriolytic activities of streptomycetes have excited considerable interest, since they are linked to some degree with bactericidal properties. Such physiological properties are outside the limits of this article, and the reader is referred to the work of Welsch (76), and of Stacey & Webb (77), for detailed accounts of the lytic system. Yet it is worth noting here, as a point of morphological interest, that lysis either appears or becomes more active when the streptomycete passes into the sporogenous phase.

Aerial mycelium.—The streptomycetes exhibit the most highly developed sporogenous phase of all the actinomycetes. The minute, abortive, aerial buds and erect, septate filaments of the nocardias, the occasional sterile hyphae which the parasitic actinomycetes may send up in an atmosphere of reduced oxygen tension, here have become sporophores, varying in complexity from simple filaments to elaborately branched and coiled, or whorled, aerial hyphae, all capable of bearing conidiospores. The analogy here

with certain fungal fructifications is very marked and has led to much taxonomic confusion, which will be discussed in the final section.

As Ørskov (7) and Jensen (5) have demonstrated, the initial steps in the development of the aerial branches of the streptomycetes are exactly the same as in the other groups of actinomycetes. The first visible stage shows a refractile granule, a bud, outside the vegetative hypha. This bud then elongates into a filament, which becomes more or less branched according to the species, strain, and nature of the medium. Aerial filaments may arise from any vegetative hypha, but tend to appear in the first instance at the centre of the colony, where the vegetative growth is at its densest and metabolic activity at its highest.

Thus is obtained the typical round, mat-like colony, firmly anchored to the substratum by vegetative hyphae, which give it a radiate appearance, and bearing a powdery crown of sporogenous hyphae. Where the plate is very thinly seeded, and the nutritive conditions are favourable, one or two colonies may achieve dimensions of 2 to 3 cm. in diameter; they may then gradually develop secondary rings of aerial mycelium, which arise from the submerged vegetative hyphae at a uniform distance from the initial growth; eventually they may present the aspect of "Liesegang rings" composed of alternating sporogenous and vegetative phases. This is certainly reminiscent of certain fungi, and one remembers that the old trivial name of the actinomycetes as a whole was ray-fungi. Yet, as all bacteriologists know, streptomycetes which appear as air contaminants on bacterial plates grow in circumscribed colonies like eubacteria, although the sporogenous hyphae on a single colony 1 mm. in diameter may carry thousands of mature spores. In other words, they do not spread irregularly, as sporing fungi do in similar circumstances.

This inherent tendency of the vegetative mycelium of streptomycetes to proliferate radially, in stationary liquids and on nutritive solids presenting a plane surface, renders difficult the determination of the point of origin of the majority of the aerial hyphae in ordinary growths. Colonies rapidly become too dense for a clear view to be obtained except at the periphery. Nevertheless, by the use of starvation media such as tap-water or soil-extract agar, and by the careful observation of the air-liquid interface of hanging drop cultures, it is possible to follow in the living organism the

early stages of aerial development. The majority of the workers, who have gained an intimate knowledge of the growth habits of streptomycetes by means of these and similar techniques, agree that aerial hyphae may arise anywhere along the length of a vegetative filament; sometimes at a node, where branching has taken place, but also at undifferentiated portions of the filaments between branches. Examination of the growth of wild streptomycetes in soils confirms this. As Starkey (69) has pointed out, the loose straggling vegetative growth indicates the poverty of the nutrients available in their natural competitive existence. Cholodny slides or buried cover slips present a nonnutritive plane surface, against which are impressed both the vegetative filaments irregularly growing round the soil crumbs and also the sporogenous hyphae developed in the minute air pockets. Erikson (64) illustrates a representative growth, which demonstrates clearly that the monopodial branching, which is the rule in the vegetative mycelium, is also the starting point of the aerial mycelium. This is confirmed by Carvajal (116).

A radically different theory of the origin of the aerial mycelium has recently been put forward by Klieneberger-Nobel (62). She states that aerial hyphae appear only where "two parts of one filament or two different filaments touch each other" and presumably unite, so giving rise to fusion bodies or "initial cells," which in turn sprout and subdivide to form a "secondary mycelium." This interpretation has such far-reaching significance that it deserves careful consideration.

In the first place, hyphal fusion is not commonly encountered among the actinomycetes as a whole. Indeed, a general absence of anastomosis is one of the characteristic features of the group, wherein they differ most markedly from fungi. The writer has watched literally thousands of growing colonies under a great variety of cultural conditions, and it has been most instructive to note the way in which, when one filament comes in contact with another, it slides over, under, or around the obstruction. This can be observed especially well when a dilute suspension of spores is seeded on moist cellophane over a petri dish containing a substrate which does not favour dense vegetative growth. Under such conditions the hyphae of adjacent growing colonies extend simultaneously towards one another. The distribution of dialysable nutrients is more or less even over the plate, and the thinness of the

film of moisture restricts to some extent growth in different planes. The apparently greater dimensions of the cells, due to the refractivity and permeability of the cellophane (42), is also a help. In no circumstances has any fusion been detected between filaments of the same or of different colonies. However, Carvajal (116) in electron microscope studies noted fusion between germ tubes and filaments of young *S. griseus* cultures.

Secondly, the technique employed by Klieneberger-Nobel (62) has certain drawbacks. Although the stained cover slip growths provide excellent pictures of germinating spores and of young mycelia, such fixed preparations give an erroneous representation of subsequent three dimensional mycelial growth. As has been noted earlier, the dominant characteristic of the streptomycete colony, looked at in the flat, is its radiate pattern; but, looked at from above or below in a liquid medium, it may be spherical, hemispherical, or ellipsoid. Branching takes place simultaneously in all directions in the liquid surrounding a small agar block on a cover slip. When fixed and dried, short and closely attached branches stick together, and so produce the "nests" she describes. On the other hand, filaments often elongate and follow in a sinuous fashion, without much lateral branching, the narrow channels or bays of liquid which form on a cover slip as the film of moisture retracts or moves its position slightly. In such a way can "scrolls" and "loops" be produced in the vegetative mycelium. All these phenomena are variable and temporary, due to the physical conditions of growth, and without any structural significance. Yet, when fixed and stained preparations only are examined, that fact is apt to be overlooked. Finally, the filaments are so slender, usually less than one micron in diameter, that it is often exceedingly difficult to be quite certain of the relative position in life of small stained sections of closely interlaced filaments. The hypothesis of fusion cells is so important that it should be attested by various workers using different techniques before it can be accepted. It is not sufficient to say that "though it was not possible to follow up the process of fusion itself, both the 'nest' formation and the structure of the secondary mycelium favour such an assumption" (62).

Sporulation of the aerial mycelium.—Drechsler (78), who studied the structure of the sporulating aerial mycelium by means of stained impression preparations, endeavoured to classify the organisms in accordance with the mode of branching: slightly

branched, much branched, whorled, bearing sinistrorse or dextrorse spirals. Waksman (79) and subsequent workers have found that these features depend to such an extent on the nature of the medium that they cannot be used as rigid diagnostic criteria. Yet it is true that while a species such as *S. coelicolor*, which generally produces spirals, may on some media produce only straight branches, other species such as *S. griseus* never produce spirals. The production of spirals appears to represent the highest stages of complexity in the development of the aerial mycelium of the streptomycetes. The conidiospores formed in spirally wound hyphae are in general spherical to elliptical, and uniform in size; whereas in straight hyphae they are cylindrical, and more often subject to irregularities in size. Jensen (5) notes that the irregular cylindrical spores of certain *S. albus* strains show the nearest approximation to the cells produced by the straight aerial filaments on the firm-textured nocardias. As Krassilnikov (25) has pointed out, nocardias never produce spirals.

Even before sporulation commences, the aerial hyphae of streptomycetes present constitutional differences in structure from the vegetative filaments. They are broader, a fact observed by most workers, and their cell walls also appear thicker. This latter point has been demonstrated by Plotho (80), using a variety of staining methods, and also by Erikson (81), using Sudan IV and other fat-staining techniques. Carvajal's electron micrographs (116) show fragments of transparent film adhering to the exterior of mature spores. Erikson's demonstration (81) of the lipid nature of the outer layers of the aerial filaments is of interest, since it appears to be correlated with the hydrophobe property which is shown by these hyphae in most varieties of streptomycetes. A rare exception is the species named *S. hygroscopicus* by Jensen (5) on account of its moist smeary aerial mycelium. The widespread occurrence of this protective waxy membrane in the sporogenous hyphae of the streptomycetes is shown by the powdery "bloom" so characteristic of the aerial mycelium. It may also be one of the reasons why the spores are so markedly resistant to desiccation, a phenomenon noted as long ago as 1895 by Acosta (82).

There has been and still is much controversy concerning the actual mode of subdivision of the aerial mycelium, which is attributable in part to the fine dimensions of the structures concerned and the celerity with which sporulation proceeds in any one hypha

under investigation, but also perhaps to natural differences among the species. Lieske (48), Ørskov (7), Jensen (5), and Erikson (24) have observed unstainable intervals in the hypha before external changes take place, and separation of the protoplasm without formation of primary transverse walls. Drechsler (78) drew beautiful pictures of primary septa splitting into halves, which few other workers have seen. Lachner-Sandoval (65), Sauvageau & Radais (83), Plotho (80), Carvajal (116), and Klieneberger-Nobel (62) have observed primary septa. The last three workers have published excellent photographs. It is interesting, however, that Plotho (80) found septa in only two strains with cylindrical spores ("segmentation spores") and accepts the general interpretation of condensation of the protoplasm and constriction of the walls, as given by Lehmann & Neumann (84), for the remainder of her strains. It is even more noteworthy that with the techniques she employed the vegetative mycelium remained nonseptate, whereas Klieneberger-Nobel (62) used the same tannic acid technique to demonstrate transverse walls in both aerial and vegetative mycelia. Carvajal's electron micrographs (116) revealed septa in almost all the sporogenous hyphae of *S. griseus*. Further investigations on the nature and constitution of internal membranes seem necessary before this conflicting evidence can be resolved.

The other point at issue is the presence of "nuclei" in the sporulating hyphae. Rippel & Witter (85) and Schaede (86) obtained diffuse staining with Feulgen's method. On the other hand, Badian (87), Plotho (80), Klieneberger-Nobel (62), and Carvajal (116) have given strong support to the conception of regularly disposed and quantised chromatinic material. The reviewer, who has applied the various methods of nuclear staining to a number of species, is unable to come to any decision on the matter, but is more inclined to agree with the findings of Rippel & Witter (85). Thus, it has been found that spores which, by Klieneberger-Nobel's technique (62) show a large round "nucleus," when stained by the classic iron-haematoxylin method present a characteristic bipolar appearance. Carvajal (116) found both uni- and multinucleate spores. In this connection, the criticism of Knaysi (88) concerning the similar structures demonstrated by Robinow (63) in eubacteria is relevant:

The possibility that the observed bodies are nuclei can not be doubted—but the problem does not involve only demonstration of bodies which stain as nuclei,

but also proof that such bodies were not formed by fixation or hydrolysis with acid from nuclear material not differentiated in the living cell as nuclear bodies; it also involves demonstrating that the bacterial cell contains no other bodies which stain like nuclei.

There can, however, be no doubt that there are many constitutional differences between the sporulating and the vegetative hyphae, which will repay further study. To take only a few instances at random: Aoki (89) found that the spores of the six strains he tested serologically possessed a more complex mixture of antigens than the vegetative filaments. Schatz & Waksman (90) noted that streptomycin was not produced by an asporogenous variant of *S. griseus*. The bacteriolytic activities of several streptomycetes have frequently been correlated with the appearance of the aerial mycelium (75, 91). On the other hand, lysis of *S. griseus* by an actinophage was complete only when the inoculum consisted of spores (92).

Asporogenous variants.—Since Lieske's elaborate presentation (48) of the facility with which the streptomycetes can produce in artificial culture sectors, which vary in pigmentation and in presence or absence of aerial mycelium from the dominant and parent cultures, there have been occasional references in the literature to this phenomenon, but no satisfactory explanation thereof. Most of the reported variants obtained by subculturing from aberrant sectors have been fluctuating or "dauermodifikations," which on continued observation under different cultural conditions give rise to the parental form again. For the same reasons as those given by Christensen *et al.* (93), when discussing sectors and patches in phytopathogenic fungi, viz., "the lack of precise knowledge of cytological conditions such as single spores or hyphal tips," it has been difficult to resolve the question of the mutant nature of variant colonies derived by mass subculture from sectors.

Jones (94, 95) has amply demonstrated the stability of streptomycete cultures maintained in sterile soil. This has been confirmed by Erikson (64), who also noted with a large range of soil organisms that, when first isolated on a simple substrate such as soil extract agar, all the colonies were capable of producing aerial mycelium. The change in the mode of growth induced by artificial cultivation—radial proliferation, an enormously increased bulk of cellular material compacted together in a small space, yet still retaining filamentous continuity—must certainly have far-reaching

effects upon the metabolic activities of the organism and upon the ability of certain isolates to reproduce themselves by sporogenous hyphae. It is tempting to speculate whether some of the desired information on this point might not be gained, if the technique applied by Gale (96) to streptococci could be so modified as to determine the effect of mycelial density upon the passage of amino acids across the cell walls of individual filaments.

Thus, the general observation that excess of nitrogen in complex media tends to favour vegetative at the expense of aerial growth is fairly well established (64, 97). Yet Jones (95) noted a soil strain which lost its ability to form aerial hyphae after a few transplants on glycerol-nitrate agar, while Appleby (26) has described an asporogenous variant of *S. griseus* appearing in simple liquid cultures, capable of growing on a variety of inorganic substrates, whose frequency was increased by ultraviolet irradiation of the spores of the parent culture. As the result of observing the variations in physiological properties such as pigmentation shown by his asporogenous strain, Jones (95) concluded that "sharp and considerable differences may occur in the structurally undifferentiated (vegetative) mycelium." In the case of Erikson's studies (27) with a number of *S. coelicolor* strains, sporeless sectors and colonies appeared from a vegetative inoculum only when this was aged or degenerate. Vegetative mycelium frequently passed through liquid cultures of different composition was on the whole stable. Jones (95) also noted the loss of certain properties following prolonged cultivation on one medium, and their restoration, at least in part, when the organism was transferred to other media or especially to soil. It is worth remembering that both Rosebury (52) and Erikson (43) have stressed the beneficial effects of the "mixed diet" method of cultivation upon the vegetative growth of the most delicate and short-lived of all the actinomycetes, the parasitic *A. israeli*.

Variation in monosporous lines.—Stanier (72), viewing the extraordinary variety of variously pigmented, sporulating, sporeless, agar-liquefying, and nonagar-liquefying colonies produced by *S. coelicolor*, was of the opinion that variation must be due to the process of conidium formation. Although there are still many difficulties in the way of an unequivocal interpretation of the cytological findings of Badian (87), Carvajal (116), and Klieneberger-Nobel (62), as mentioned earlier, there is certainly a growing body

of evidence in favour of the existence of constitutional differences not only between vegetative and aerial mycelium, but also between different aerial filaments, and the several conidia of one filamentous chain.

The variations in pigmentation and sectoring reported by Schaal (98) for *S. scabies* appeared in single cell strains; this was also the case with the physiological differences noted by Jones (95). Because of the sensitivity to environmental reaction displayed by its red-blue indicator pigment, *S. coelicolor* is an excellent test organism. The work of Cochrane & Conn (99) and of Oxford (100) has made clear the causal relationship of pH to pigmentation when the inoculum is mixed vegetative and aerial growth or spore suspensions. Yet that certain spores are capable of producing achromogenic variants, independently of the nature of the medium, has been proved by Erikson (27), who cultured under identical conditions the sister-spores of different conidial chains. Thus, out of eight spores dissected from one chain in a healthy, well sporulated colony, six reproduced the characters of the parent, while two developed colourless variants as well. These variants were also characterised by a loss of the agar-liquefying property of the strain, and by differences in the nature of the aerial mycelium.

This lack of genetic uniformity in the sporogenous elements of one filament could be accounted for by Carvajal's observation (116) that spores may have one or more "nuclei," and that "the number of nuclei is by no means always proportional to the size of the cell." Granting that chromatinic bodies are embedded in the cytoplasm, the writer has frequently observed an irregular disposition of this material in the separate elements of sporing hyphae, as revealed by vital staining with methylene blue. There are also always some spores which seem empty and do not stain at all, as Carvajal (116) has mentioned, and they may occur at any position in the chain. Erikson (27) found that several of the spores dissected from the hyphae produced in a vigorous aerial mycelium were nonviable, and also noted the fairly frequent occurrence of simultaneous germination of two or more spores while still mutually attached. Again, Carvajal (116) has illustrated two germ tubes arising at right angles from the narrow isthmus connecting two spores. These observations indicate possible ways in which disparate portions of the reproductive filament may develop and give rise to variants.

Morphotypes and biotypes.—The concept of "physiological races" in the eubacteria has proved most fruitful in its manifold applications, while a knowledge of the biotypes established among the rust fungi has been of incalculable practical advantage. Some of the most interesting results of the intensive studies now being carried out with *S. griseus* are those which illustrate the natural existence of a variety of morphotypes and biotypes. Thus, Waksman *et al.* (101) state that in organisms freshly isolated from soil certain strains show variations in the nature of the aerial mycelium, pigmentation of vegetative growth, capacity to produce antibiotic substances, and preferential ability to grow under submerged conditions of culture. Carvajal (102) has noted the characters of various biological races; and it is only to be expected that from the large mass of work directly inspired by Waksman's discoverv of streptomycin there will emerge a definitive structure of *S. griseus*, which will be of help in elucidating the nature of other streptomycetes.

In particular, the use of an actinophage in identifying streptomycin-producing strains of *S. griseus* (92) has many possible applications. Dmitrieff & Souteeff (71) noted an actinophage in their cultures of an organism obtained from morbid tissue in man, but did not succeed in isolating it. As Woodruff *et al.* (103) remark,

> the accumulative generations of growth of the cultures constantly subject to chance contamination through faulty air filtration or insufficiently sterile laboratory and plant equipment, and through errors in techniques have made it highly probable that actinophages would be rediscovered for actinomycetes.

Such discoveries must also in time yield results which will have a bearing on our knowledge of the gross morphology of the organism and of its cellular elements.

GENUS: MICROMONOSPORA

This is the least known group of the actinomycetes, and morphologically the most stable. Again, it is from the pioneer studies of Ørskov (7) and Jensen (104, 105), that our basic knowledge of the genus is derived. The vegetative mycelium is similar to that of the streptomycetes, although the filaments are in general more slender, 0.3 to 0.8 micra thick, and more uniform. True septa are very rarely found in the vegetative filaments, which branch in the customary monopodial fashion of the actinomycetes. Growth on the whole is slow, and the individual colonies never attain the

dimensions of streptomycetes, when growing free from competition under suitable nutritive conditions. Yet the most vigorous species, such as *M. chalceae* and *M. fusca*, when streaked from massive spore suspensions develop an abundant mycelial mat of growth. The fine photographs published by Waksman *et al.* (106) of thermophilic micromonosporae in composts illustrate a loose straggling development of the vegetative hyphae similar to that displayed by streptomycetes in soils.

The few instances of aerial mycelium that have been reported (24, 105 to 108) are all of the nocardial type, a sparse development of mostly unbranched and undivided filaments. Such development is spasmodic and infrequent and does not represent a sporogenous phase. Reproduction takes place by means of small spherical to oval spores, which are produced singly on the distal ends of short lateral branches of the vegetative mycelium. These spores are non-acid-fast, gram positive, and highly refractile. Jensen (105) has drawn attention to the similarity between this method of spore formation and the budding of the pear-shaped or coccoid cells in nocardias, and, indeed, to the early stages of vegetative branching in all the actinomycetes. If, as has been said earlier with regard to bacteria in general, branching is only a specialised form of budding, then there are valid grounds for accepting Jensen's contention (105) that it is only a further step for such club-like branches to develop into definite spores of reproductive capacity.

So far no detailed cytological studies of these small spores have been made, yet it is apparent from the studies of various workers that constitutional differences do exist between the vegetative hyphae and the spores. Erikson (108) has demonstrated that differential staining can readily be effected by several techniques, the spores invariably appearing brilliant against the counter-stained vegetative mycelium. Although in mass they have a moist glistening appearance, quite different from the dry aerial spores of the streptomycetes, they are also resistant to desiccation (7, 104, 108). Perhaps their most striking characteristic is their intense refractility, which in the writer's experience is more marked than in the case of any other actinomycete cell element. This is especially conspicuous in all darkground preparations. By means of Pijper's sunlight-darkground technique (109) the spherical spores of *M. fusca* have appeared dazzling, in striking contrast to the vegetative mycelium (110). Spores, which retain their light scattering

properties, have been noted *in situ* in colonies, whose internal vegetative hyphae are mainly disintegrated, after six months by Erikson (108), and after three years by Hungate (111).

Micromonosporae are remarkable in having an organised, internal, colonial structure. Sectioning reveals a definite zonate pattern of sporulation. This was noted by Erikson (108) for a number of aerobic strains grown on simple synthetic media, and confirmed by Hungate (111) for his anaerobic strain grown on cellulose agar. The persistent subsurface sporulating zone has been described (108) as a mechanism which may facilitate the survival of the organism through North American winters in lake bottoms (112). It is also noteworthy that these strains are capable of utilising very resistant substances such as chitin and lignin (108). One of the most interesting facts noted by Hungate (111) was that his cellululose-digesting organism, isolated from the gut of a wood-eating termite, exhibited a characteristic propionic fermentation. This has been advanced in support of Stanier & van Niel's theory (113) that the actinomycetes originated from the propionic acid bacteria. From the diverse and somewhat unrelated data available, it is clear that we are only at the first stages in our knowledge of this section of the actinomycetes.

The reviewer cannot let pass this opportunity of remarking that in erecting a new species for a micromonospora-like organism, on the grounds of partial segmentation of the vegetative mycelium and infrequent production of spores, Erikson (24) erred. This was later admitted, when the writer had occasion to study a number of undoubted micromonosporae (108). The organism having now reached sanctuary under the name of *S. gallicus* among the streptomycetes in *Bergey's Manual* (2), it has been thought advisable to mention the matter again.

TAXONOMY

Even without sharing Huxley's confident belief (114) that "the problem of systematics, regarded as a branch of general biology, is that of detecting evolution at work," it is always permissible to hope that no scheme of classification will be permanently adopted which separates clearly related forms by artificial barriers. The scheme for the actinomycetes propounded by Waksman & Henrici (6), and adopted by Bergey (2), is in its main divisions the soundest yet put forward. In the preceding pages an attempt has been

made to show that, although the finer morphological details may be a function of the environmental conditions affecting the organism, there does exist a fundamental ground-plan of structure which is discernible in each group. The consistent production of a vegetative mycelium; the spontaneous subdivision of that mycelium, or its coherence as a unit; the erection of aerial buds, filaments, and eventually of complexly branched and coiled, constitutionally differentiated sporophores; the development from ordinary mycelial branches of single refractile spores; all these are permanent features in the growth of certain naturally occurring groups of microorganisms. The outstanding merit of the proposed classification is its acceptance of these facts. Its chief defect is the separation of the parasitic, anaerobic *Actinomyces* from *Nocardia*, in spite of the morphological similarities between the two.

The use of pathogenesis and anaerobiosis as distinctive criteria for the separation of genera is gradually being recognised as unsound. Thus, one is rejoiced to see that in the latest Bergey (2) *Phytomonas*, that artificial collection of plant pathogenic forms, is no more. Moreover, delimitation by means of these two criteria has not been carried to its logical conclusion. Among the aerobic nocardias there are undoubted pathogenic forms, such as *N. farcinica*, the type species; while there is at least one anaerobic species among the saprophytic micromonosporae. It is also a trifle incongruous that the historic term *Actinomyces* should now be limited to the two species, *A. israeli* and *A. bovis*, which, being of such specialised occurrence, are little known to the majority of bacteriologists; whereas the ubiquitous actinomycete of soil and air, one of the most common laboratory contaminants, has to acquire an entirely new name, *Streptomyces*.

It is only fair to add that the authors of the scheme have recognised these and similar criticisms put forward by Baldacci (19) and others. As long as we are bound by the Botanical Code, the term *Actinomyces* must evidently be applied to the organism of "lumpy jaw"; and *Nocardia* Trevisan 1888 has undoubted priority over *Proactinomyces* Jensen 1931. The fact that the aerobic nocardias and anaerobic actinomycetes can be sensibly and logically grouped together in *Proactinomyces* (Ørskov's Group IIa and IIb) is admitted, but the laws of priority do not permit it. Against such a deadlock it seems impossible to proceed. It is best to accept, with the reservations mentioned, the new scheme of classification and

its generic names. The reviewer, once an eager "splitter," has been disciplined by a study of the actinomycetes into a resigned "lumper."

There remains only the necessity to say a word in support of Waksman's concept (60) of species-groups, as allowing a much greater degree of freedom in the definition of individual organisms. The extreme sensitivity with which all the actinomycetes react to environmental conditions demands a certain liberality of interpretation in the assignment of specific characters. To turn over the long lists of insufficiently described species in *Bergey's Manual* is to think, not perhaps without a guilty sense of responsibility, that it is surely time that microbiologists, as well as palæontologists, "built up a body of public opinion which should discourage the '*nom. nov.* man' by making him feel the pest he is" (115).

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CILIATED PROTOZOA: CYTOGENETICS, GENETICS, AND EVOLUTION[1,2]

By T. M. Sonneborn

Department of Zoology, Indiana University, Bloomington, Indiana

This review on ciliated Protozoa deals with recent literature on several aspects of cytogenetics, genetics, and evolution. The cytogenetics section includes: chromosome cytology; the mechanisms by which meiosis and fertilization are induced and carried out; the behavior of nuclei and cytoplasm during conjugation; the structure, division, and significance of the macronucleus; and genetic materials in the cytoplasm. The genetics section includes: racial differences; mating types and the breeding system in *Paramecium;* the physiology and genetics of "killer" paramecia; and determination, transformation, and inheritance of antigenic traits in *Paramecium.* The evolution section includes only a brief account of the work of Gause (25) and his collaborators on adaptation and mutation in relation to evolution in *Paramecium* and *Euplotes.* As some of these subjects were reviewed not long ago by Wenrich (98), Sonneborn (80), and Harrison (35), the present review deals mainly with work of the past two years.

CYTOGENETICS

Cytogenetics of the ciliated Protozoa is in a confused condition. This is due only in part to the difficulties and peculiarities of the material. It is also partly due to the espousal of views that seem untenable because of their incompatibility with knowledge of general cytogenetics or knowledge of genetics of the ciliated Protozoa themselves. For example, the literature includes many accounts of transverse chromosome division, of fewer chromosomes at ordinary mitoses than at the meiotic nuclear divisions of the same species, and of chromosome reduction at the second meiotic division. These dubious interpretations are widely accepted. On the other hand, some papers that have appeared during the past few years herald a more consistent point of view. The paradoxical nature of the accounts in the literature and the necessity

[1] This review covers the period from January, 1946 to January, 1949.

[2] Contribution #407 from the Department of Zoology, Indiana University.

for a rational integration of genetics and cytology are being recognized.

Chromosomes and Mitosis

An outstanding contribution is the report of Chen (15) on chromosomes of Opalinidae. Chen's study demonstrates that the chromosomes of *Zelleriella* are in detail comparable to the chromosomes of higher organisms. Each of the 12 chromosomes of a haploid set has been shown to be unique and constant in its morphology. Certain definite chromosomes bear nucleoli and the relation of each nucleolus to its chromosome has been described in minute detail. Prior to the work of Chen, chromosome cytology of the Opalinids had been as confused as the cytology of any other Ciliate. His thorough and careful study shows that Opalinid cytology agrees with general cytogenetics.

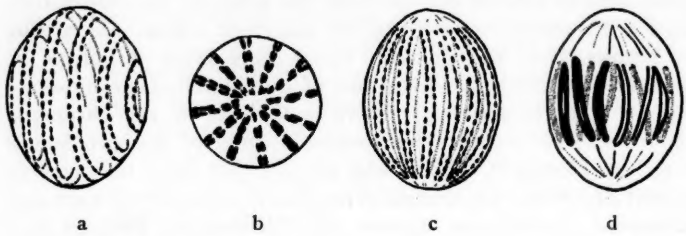
Unlike the Opalinidae, which are almost alone among the Ciliophora in possessing a single type of nucleus, the Ciliata of other groups usually possess two kinds of nuclei, macronuclei and micronuclei. The macronucleus is particularly puzzling and will be dealt with later. The micronucleus is generally considered as comparable to a germ-line nucleus in higher organisms. It alone undergoes meiosis and participates in fertilization. Accounts of the morphology and behavior of micronuclear chromosomes are, however, peculiar and paradoxical. Although often not recognized, the difficulties have been pointed out by a number of investigators.

Devidé & Geitler (19) have focused attention on one of the main difficulties. In agreement with scattered reports of other observers, they find the number of chromatic units (chromosomes?) appearing in a nucleus during the first meiotic division (for example, in *Colpidium*, *Euplotes*, *Vorticella*, *Stylonychia*, *Chilodonella*, and other genera) is larger than the number visible in the same species at ordinary vegetative mitotic divisions. In view of the established constancy of chromosome number in higher (and other lower) organisms, Devidé & Geitler hold that the chromatic elements visible at ordinary mitoses cannot be individualized chromosomes, but must be aggregates of chromosomes. Moreover, comparative studies indicate that aggregation of chromosomes may even occur to some extent at meiosis. The chief value of this paper is its attempt to generalize the observations and point out the problems.

The observations indicate that a special type of mitosis must prevail in the micronuclei of Ciliates. Not only do the chromosomes fail to appear on the spindle in individualized form, but it has repeatedly been maintained by others that the chromatic elements of the mitotic micronucleus, whether they be chromosome aggregates or something else, divide transversely. Yet genetic evidence demonstrates behond question that the genes are as a rule distributed with precise equality to the daughter micronuclei arising at mitosis. How can precisely equal segregation at mitosis be understood without the appearance of individualized chromosomes and in spite of seeming transverse division of the chromatic elements? The necessity for thorough study of this problem is urged by Devidé & Geitler, who suggest that there may emerge from it significant information in relation to centromeres, nucleolar substance, chromosome matrix, and spiralization. Perhaps here too may be the clue to the well-known relative inertness of micronuclei and to the enigmatic structure and division of the macronuclei that develop from them.

A suggestive, independent approach to these problems is given by Raabe (61) in his studies of micronuclear division in *Urostyla grandis*. Raabe finds the following main events occurring during vegetative micronuclear mitoses. At first the micronucleus stains uniformly; later the stain is concentrated in granules which become smaller and more numerous. These are then organized into a spiral ribbon extending from pole to pole, but with the spiral axis transverse to the polar axis of the nucleus. The ribbon is largely acidophilic with two rows of granules one at each edge of the ribbon. These two rows of granules are believed to be the two products of division of the chromatic equipment of the nucleus (Fig. 1a) .The turns of the coiled ribbon approach each other at the two poles and are in that region severed into segments of ribbon running from pole to pole parallel to the polar axis of the nucleus (Fig. 1b and 1c). Though they simulate chromosomes in some respects, these ribbon segments are not considered to be chromosomes for they lack individual definitiveness of structure. Nevertheless, 12 segments seem regularly to arise from the spiral ribbon. The segments condense, contracting away from the poles and occupying the middle third of the spindle (Fig. 1d). There they are believed to split longitudinally, with the two products of division passing along spindle fibres to opposite poles.

Although Raabe does not hold that all details of the cytological evidence are entirely convincing, his general account is a noteworthy attempt to relate the observations to the solution of the paradoxes mentioned above. He presents good evidence for two longitudinal rows of chromatic granules along the ribbon and its segments and for longitudinal division of the ribbon segments,



a. Spiral Ribbon Stage
b. Segmentation of Ribbon
c. Segmentation of Ribbon
d. Condensation of Ribbons Away from Poles
(b is a polar view of the same stage as c)

FIG. 1.—Micronuclear mitosis in *Urostyla grandis*, after Raabe (61).

but the lack of any evidence of centromeres raises difficult problems as to how the products of the split segments pass to opposite poles. This critical phase of micronuclear mitosis needs further investigation. But Raabe's observations go a long way towards making comprehensible the effectively equal distribution of chromosomal material without the appearance of individualized chromosomes. If the chromosomes fuse end to end in the formation of the ribbon or continuous spireme, then the orientation of the ribbon, its subsequent segmentation, and the longitudinal division of the segments make equal distribution of chromosomal material to the two poles intelligible to a considerable degree. In some respects, similar observations have been made by Colwin (17) on *Urceolaria*.

MEIOSIS AND FERTILIZATION: CONJUGATION AND AUTOGAMY

What induces the occurrence of meiosis and fertilization?—Meiosis and fertilization occur in ciliates during conjugation and, in some species, also during autogamy. The answer to the question

of what induces meiosis and fertilization therefore resolves itself into discovery of the mechanisms that bring about conjugation and autogamy. Directly or indirectly these problems have been under investigation for more than 60 years, but two important advances made within the past 12 years are leading to a deeper insight into them. First, Sonneborn (68) discovered that in *Paramecium aurelia* the individuals are physiologically differentiated into diverse sexes or mating types. When individuals of complementary mating type are brought together under appropriate conditions, they agglutinate and proceed to conjugate. Comparable phenomena have subsequently been discovered in other species of *Paramecium* and in other genera of Ciliophora. Second, Metz (50, 51) adopted and developed the working hypothesis that meiosis and fertilization are induced by a series of reactions initiated by contact between complementary substances on the surfaces of the complementary mating types.

Metz's hypothesis was built upon a number of observations. Sonneborn (68) reported that animals of complementary mating type did not attract each other, but that their union depended upon accidental contact. In agreement with this, fluid in which animals of one mating type had lived produced no effect on animals of the other mating type, but two animals of the same mating type could briefly and weakly stick to each other if one of them had recently broken loose from contact with an animal of the complementary mating type. These observations indicate that the mating reaction involves surface substances. Whole animals are not required for the agglutination reaction for this can occur between cell fragments, even enucleated fragments, of animals of complementary mating type [Tarter & Chen (94)]. Finally, Boell & Woodruff (9) made the remarkable observation that live paramecia of one mating type will agglutinate with dead paramecia of the complementary mating type.

This observation by Boell & Woodruff has been repeatedly confirmed by Metz (49) using a wide variety of physical and chemical agents for killing the paramecia. However, the mating reactivity of the animals is blocked or destroyed by a number of conditions: (*a*) killing reactive animals (*P. aurelia*) by exposure to 52°C. for 5 min. (49); (*b*) killing reactive animals (*P. bursaria* and *calkinsi*) by exposure to x-rays (105, 106); (*c*) exposure of formalin-killed reactive animals (*P. caudatum*) to 100°C. (37); (*d*) exposure

of formalin-killed reactive animals (*P. aurelia*) to specific antisera (54); (*e*) complete disruption of the killed animals (*P. aurelia*) by either physical or chemical means (49).

Metz adopted the standard procedure of mixing formalin-killed reactive animals of one mating type with live animals of complementary mating type and then proceeded to make a remarkable series of observations that opened the door to a new and important field of investigation. He found (50, 51) that the live animals in the mixture later formed tightly united pairs after having agglutinated with the dead animals. These pairs are referred to as "pseudo-selfers." They become united only at the anterior holdfast region and not at the more posterior paroral cone region where nuclei are normally exchanged during conjugation. Except for the failure of the paroral cones to unite and the consequent inability of the mates to exchange gamete nuclei, the pseudo-selfers behave like normal conjugants: they remain united for about the same length of time; they soon lose the capacity to agglutinate with cells of complementary mating type; and they undergo macronuclear disintegration and meiosis. Presumably each pseudo-selfer fertilizes itself, i.e., undergoes autogamy. After the pseudo-selfers separate, each produces a culture of the same mating type as the live animals in the original mixture. This indicates that the animals which unite in pseudo-selfing pairs are alike in mating type; at least no permanent change of mating type takes place.

In the same papers, Metz further showed that union in pseudo-selfing pairs was not necessary for the induction of meiosis and autogamy. Single live animals that had been in agglutinative contact with dead animals of complementary mating type also went through the typical nuclear changes characteristic of autogamy. The experiments show clearly that holdfast union (i.e., pseudo-selfing) is dependent upon contact with animals of complementary mating type. They indicate but do not yet show clearly whether the induction of autogamy requires such contact or whether it can be induced by substances diffusing from the dead animals of complementary type. Fluids in which paramecia have lived are indeed known in certain special cases to induce not only macronuclear breakdown and meiosis, but even abnormal pairing between animals of the same mating type (12, 41, 57). This is the special case in which the culture fluid has previously been occupied by certain types of killer cultures and contains the killing substance

paramecin. The effect of paramecin is neither mating type specific nor species specific, but may bear on the mechanism by which meiosis is induced (41).

Extension of the analysis to *P. calkinsi* (51) brought out two new points of special interest. First, autogamy was induced in live unpaired animals of this species by the same method in spite of the fact that autogamy does not occur normally in this species. Both autogamy and pseudo-selfing could be induced in living type II animals when mixed with dead type I animals. Second, neither pseudo-selfing nor macronuclear breakdown occurred in the reverse mixture of living type I animals with dead type II. Diller (22) claims to have found both autogamy in single animals and pseudo-selfing in addition to true conjugation when both mating types in a mixture were alive.

The observation that pseudo-selfing occurs in only one combination of mating types in *P. calkinsi*, and not in the reciprocal combination, assumes particular interest in view of similar results obtained by Hiwatashi (37) on four Japanese varieties of *P. caudatum*. In all four of these varieties, dead animals of one type could induce pseudo-selfing in live animals of the complementary type, but the reciprocal combination yielded no mating reaction and no pseudo-selfing. Hiwatashi made the further important observation that the live animals in the latter ineffective combinations conjugated, i.e., underwent selfing, two or three days later.

This observation raises a theoretically important question. Has exposure of live animals to dead animals of complementary mating type induced autogamy, followed by change of mating type, or has it induced selfing by some other mechanism? Previous work indicates that selfing may occur in clones of *P. caudatum* either without autogamy and without permanent change of mating type (31) or with autogamy followed by change of mating type (30). It is not known which of these two known types of selfing took place in those cultures of Hiwatashi that selfed some days after contact with dead animals of complementary mating type. Further analysis of the matter is desirable for it would be theoretically important and might throw light on the failure to obtain pseudo-selfing in one of the combinations of dead and live mating types in *P. calkinsi*.

The induction of meiosis has recently been studied further by

Metz & Foley (53) with the aid of an aberrant culture (CM) that can give the agglutinative part of the mating reaction, but cannot proceed to fuse and conjugate. Such cultures had been found previously and had led to the conclusion that agglutination and fusion were distinct and separable parts of the mating reaction (13, 71), a conclusion justified also by the behavior of the CM culture.

From this culture both complementary mating types of variety 4 were isolated. They agglutinate with each other and with the complementary normal mating types of variety 4. However, the CM animals involved in such agglutination reactions never join with each other or with normals in pseudo-conjugation nor do they undergo the nuclear changes of autogamy, although normal autogamy does occur periodically in CM animals. On the other hand, normal live animals that have agglutinated with CM animals do undergo the nuclear changes of autogamy, either as isolated individuals or as pseudo-conjugating pairs. The same results are obtained regardless of whether both types of animals in a mixture are alive or whether one of the two is dead. Finally, although the CM animals cannot be induced to undergo autogamy by contact with normals, they do undergo periodic autogamy in the absence of such contacts.

On the basis of his observations, Metz (51, 52) draws a number of important conclusions. (*a*) Surface contact between normal sexually reactive animals of complementary mating types involves interaction between complementary mating type substances on the surface of the animals, probably on their cilia. The interaction of these substances is held to set in motion a chain of reactions leading to the various parts of the processes of conjugation and autogamy. The report of Metz & Fusco (54) indicating that the whole chain of reactions can be inhibited by exposing dead reactive animals to certain anti-Paramecium sera supports the idea of a surface initiation of the reaction chain. (*b*) The reaction chain is held ultimately to branch into five side reactions: holdfast formation, loss of agglutinative reactivity, paroral cone formation, meiosis and fertilization, and macronuclear disintegration (Fig. 2). (*c*) In the CM stock, the chain of reactions is blocked before any of the five side reactions branches off. (*d*) Natural autogamy is stimulated differently from autogamy induced by contact with dead animals; the unknown stimulus to normal autogamy enters

the chain of reactions between the CM block and the branching of the chain. This conclusion follows from the occurrence of natural autogamy in the CM stock.

The most vulnerable conclusion is the second one, as Metz fully appreciates. He has presented little or no evidence as to whether the five "side reactions" are independent or sequential. His scheme, however, constitutes a useful working hypothesis which directs attention to the problems and invites further analysis. Can any one of the five reactions be blocked without

FIG. 2.—Metz's scheme (52) of the chain of reactions set off as a consequence of contact between paramecia of complementary mating type. For explanation, see text.

blocking the other four? Can any one of the five reactions be stimulated without inducing the other four? While these questions cannot yet be fully answered, some pertinent information is available and more can be obtained. For example, amicronucleate animals are known to "conjugate" and undergo all five of the reactions except meiosis and fertilization; hence, the other four reactions are not sequential to this one. On the other hand, there is evidence that exchange of nuclei between mates is sequential to some other process than the formation of gamete nuclei themselves (14). As an example of data bearing on the other question it seems that macronuclear breakdown can occur without pairing or meiosis (20). Additional significant information may come from study of more cultures comparable to the CM culture, but blocked at a different point in the chain of reactions. The CM culture, like the similar one found earlier by Sonneborn (71), arose in later generations following interracial hybridization. A systematic search among the progeny of racial and varietal hybrids may yield other similarly valuable materials.

Meanwhile, as Metz (51) has pointed out, the scheme in Fig.

2 might be more correct if the reaction leading to holdfast union branched off from the main chain between the CM block and the stimulus for natural autogamy, for natural autogamy is not accompanied by holdfast union. Probably other features of the scheme will have to be modified as knowledge grows.

These minor criticisms in no way lessen the value of Metz's beautiful analysis. Indeed it has implications that extend far beyond his initial purpose of obtaining evidence pertinent to the fertilizin problem. It can serve as a model of how to pursue analysis of the chains of reactions involved in all the developmental processes of *Paramecium* and comparable organisms, by employing races or cultures that differ from the normal in some developmental process. That many such variants exist is indicated by the diversities in the reports of students of the cytological details of meiosis, conjugation, and post-zygotic reorganization. Some of these diversities are described in the following sections.

What nuclear events occur during conjugation?—It has long been known that the major events of conjugation are: (*a*) two meiotic nuclear divisions resulting in reduced nuclei; (*b*) an equational third nuclear division, yielding gamete nuclei; (*c*) union of gamete nuclei to form a synkaryon; (*d*) development of new micronuclei and macronuclei from the synkaryon; and (*e*) disintegration and disappearance of the original macronuclei. Each of these five major events, however, is subject to many variations in detail in different species and even to some extent within a single species.

Reduction of the number of chromosomes has been held by Finley (24) and others to take place at the second meiotic division in Ciliata, although in other organisms it regularly takes place at the first meiotic division. Before accepting such an exceptional interpretation for Ciliata, critical decisive evidence is required and alternatives must be clearly excluded. Sonneborn (80, p. 346) suggested that the tetrads may separate completely into dyads before the stage usually considered to be late prophase of the first meiotic division. This could readily be misinterpreted as indicating second division reduction. Rosenberg (64) in fact reports "as low as 28" chromosomes in an early first meiotic spindle of *Opisthonecta* and "up to 58" chromosomes on a later first meiotic spindle. However, unless the "late prophase" is really an early anaphase, there arises the problem of how homologues manage to migrate

to opposite poles. Nevertheless, there are other objections to Finley's view of second division reduction. The first meiotic division in Ciliata, as in other organisms, is a long, complicated, and unique division, indicating that it is the heterotypic or reductional division. Finally, the work of Devidé & Geitler (19) discussed earlier shows that arguments based on "chromosome" counts must be cautiously interpreted in view of the occurrence of chromosome aggregates even at meiosis.

Instead of the usual three prezygotic nuclear divisions, Finley (24) reports only two in *Vorticella microstoma*. Correlated with this omission of the third division, fertilization is unilateral, the mates are unequal, and the microconjugant does not survive. However, according to Colwin (17) three prezygotic nuclear divisions usually occur in *Urceolaria* in which also the mates are unequal and the microconjugant fails to survive. But here fertilization is reciprocal in spite of its lack of consequences in the microconjugant. The third division, resulting in two gamete nuclei, is thus seemingly an adaptation providing a means for reciprocal fertilization. Occasional absence of this third division in species normally manifesting reciprocal fertilization and equal conjugants, such as *Paramecium trichium* (21), serves to emphasize that this characteristic is sufficiently variable to be selected readily one way or the other in the course of evolution.

While reciprocal fertilization is the rule in species in which both mates survive, failure to exchange gamete nuclei may occur exceptionally. Thus Horvath (38) reports regular failure of a gamete nucleus to pass from a normal to an amicronucleate mate during conjugation in *Kahlia*. Even under these conditions gamete nuclei may behave normally in other species (10, 46). Of special interest are Wichterman's observations (99, 101), by means of the microcompressor, on living conjugants of *P. bursaria*. In some pairs of conjugants, the migratory gamete nucleus wanders from one mate, but not from the other. This leaves only one reduced gamete nucleus in one mate, but provides three that fuse into one in the other mate. This observation by Wichterman directly confirms one of Chen's suggestions (11) as to how polyploidy arises in this species. Diller (21) reports occasional failure of exchange of gamete nuclei in *P. trichium*. On the other hand, the evidences from direct observation on living conjugants (100), from the usual stained preparations (10), and from genetic studies (80) show that

exchange of gamete nuclei and reciprocal fertilization are the usual normal features of conjugation in *Paramecium.*

Unlike the gamete nuclei, the macronuclei and their disintegration products are normally not exchanged between conjugating mates. However, Diller (21) reports that strands of the disintegrating macronucleus sometimes pass across from one mate to the other in one race of *P. trichium.* Neither this nor certain other peculiarities described by Diller was found in another race of the same species examined by Dippell (unpublished data). Under some conditions certain races of *P. aurelia* form a cytoplasmic connecting bridge during conjugation and the spherical disintegration products of the macronuclei may pass across this bridge (73).

Behavior of cytoplasm during conjugation.—The behavior of the cytoplasm during conjugation differs in different species and races and even in the same race under different conditions. In Opalinids and Peritrichs, complete cytoplasmic fusion of mates is the rule. In many other ciliates, cytoplasmic fusion between mates does not normally occur. In *Kahlia* cytoplasmic fusion occurs only if one or both mates is amicronucleate (38, 40). There is both direct and indirect evidence of cytoplasmic exchange in *P. aurelia* (73), but there are racial differences in this respect (76). Recently, Sonneborn (unpublished data) has found that exposure of conjugants to the proper concentration of specific paralyzing antiserum prevents separation of the mates and leads to cytoplasmic fusion and exchange between them. Harrison & Fowler (32, 34) obtained serologic evidence for cytoplasmic exchange between conjugants of *P. bursaria;* they also reported exchange of symbiotic algae between the mates. Wichterman (100, 102) denied the exchange of symbiotic algae, but he was able to induce prolonged union of mates, probably with cytoplasmic fusion, by exposing conjugants of *P. bursaria* to x-rays (104, 105). Both Chen (14) and Diller (21) observed cytoplasmic continuity between mates in some conjugant pairs of *P. bursaria* and *P. trichium,* respectively. There is clearly no general rule about the behavior of the cytoplasm which is applicable to all Ciliata under all conditions.

The Macronucleus

Structure and organization.—As is well known, each ciliate ordinarily contains one or a few large macronuclei, but the form

of the macronucleus varies greatly from species to species. In a few species the macronucleus is represented by a large number of discrete small nuclei. In *Urostyla grandis* more than 100 separate parts of the macronucleus, each measuring about $7 \times 3\ \mu$, are present in each cell (62).

Feulgen-negative vesicles have repeatedly been reported in the macronucleus and they are sometimes considered to be nucleoli. They are again noted by Diller (21) in *P. trichium*, by Finley (24) in *V. microstoma*, and by Raabe (62) in *U. grandis*. Diller suggests that this material is in part extruded during the development of macronuclei from micronuclei. Finley observed a chromophilic cortex and chromophobic medulla in fragments of the macronucleus. Raabe finds that the Feulgen-negative material takes acid dyes. He noted that the number of these bodies is high in well-fed animals, low in nonfeeding animals, and therefore suggests that they have a nutritive function.

It has also been suggested (27, 28, 29, 55) that these "nucleoli" are an index of the degree of polyploidy of the macronucleus, as recently urged again by Subramaniam (92). In relation to this, Sonneborn (70, 72) showed that a single fragment of the macronucleus of *P. aurelia* could regenerate an entire functional macronucleus and must therefore contain at least one full set of genes. Since each macronucleus breaks up into many such fragments, it must contain at least that many complete subnuclei. Horvath (38) confirmed this observation on *Kahlia*. Evidences of these sorts support the polyploid nature of the macronucleus, according to Subramaniam (92).

Yet remarkably few observers have claimed to see chromosomes in the macronucleus and these claims are not convincing. Devidé & Geitler (19) point out that the chromosomal material of the macronucleus is probably present in the form of chromosome aggregates after the fashion of the micronuclear chromatin. On the other hand, Seshachar (66) and Seshachar & Srinath (67) believe that chromosomes cannot exist in the macronucleus. In support of this belief, however, they present an unconvincing argument and little or no evidence. Diller (22) is among the few who think they can see chromosomes in the macronucleus.

Division.—Raabe (62) describes the process of macronuclear division in *U. grandis*. At the time of cell division, the numerous discrete macronuclear parts round up and fuse into a single large

nucleus. This then undergoes a series of about eight successive divisions, the cell dividing after the third or fourth macronuclear division and each daughter cell receiving half of the eight or sixteen nuclei then present. This mechanism of fusion assures distribution of approximately equal numbers of macronuclear parts to the two daughter cells and stabilizes the number per cell at an approximately uniform level. The important discussion by Preer (57) of the consequences of random distribution of cell particles applies here with full force. The stabilizing mechanism in *Urostyla* prevents the production on a large and disastrous scale of individuals with too few or too many macronuclear parts.

Prior to division, the macronuclear parts in *Urostyla* show the characteristic band often interpreted by American workers as a "reorganization band." Raabe rejects this interpretation and holds that the band serves a mechanical function in facilitating union of the many small nuclei into one. It is difficult to see how this interpretation can be general in view of the fact that the band also appears in species with a single large macronucleus.

During division, the chromatic granules are arranged in parallel rows directed towards the poles. Because of this and the genetic requirement for effectively equivalent products of division, Raabe stresses that the term "amitosis" is misleading for this sort of nuclear division.

The macronucleus as a somatic nucleus.—The macronuclei have long been interpreted as somatic nuclei because they do not participate in meiosis or fertilization but disintegrate and disappear at that time. Horvath (38) believes his studies on *Kahlia* disprove this. His chief evidence is derived from nuclear behavior during conjugation between two amicronucleate animals. Small nuclei arise, presumably derived from the macronuclei, and these seem to give rise to the new macronuclei; but the animals remain amicronucleate. Apparently Horvath believes these small nuclei are micronuclei, but his criteria for distinguishing between micronuclei and small fragments of the macronucleus are not clear. This obscurity may also be the basis of his report that micronuclei grow directly into macronuclei following exposure to ultraviolet irradiation or to excessive quantities of bacteria (39). In any case, the failure of the small nuclei (that arise in amicronucleate animals) to participate either in meiosis or in fertilization shows that his

observations are irrelevant to the question of whether the macronucleus can take over activities of germ-line nuclei.

Cytogenetic Components of the Cytoplasm

In *P. aurelia*, the genetic studies of Sonneborn (73, 77, 78, 79) led to the conclusion that "killer" strains of this species contained in their cytoplasm a self-multiplying genetic factor, "kappa." Preer (57, 58) developed elegant methods for calculating the number of kappa particles per killer cell and the size of the particles. X-ray inactivation studies indicated that the size of a kappa particle lay within the range 0.3 to 3.0 μ. Meanwhile, van Wagtendonk & Zill (97) and van Wagtendonk (95, 96) gave evidence that the peculiar product of killer paramecia (paramecin) was a desoxyribonucleoprotein and suggested that kappa was probably chemically similar to paramecin. Preer (58) thereupon concluded that the kappa particles should be microscopically visible with the Feulgen stain and in this way he brilliantly demonstrated them to be approximately of the predicted size and number. Later he also succeeded in staining kappa by the Giemsa technique (59). The presence of desoxyribonucleic acid in kappa was further proved by enzyme digestion studies (59). The kappa particles in different races of killers were found to differ in size, form, and number. Increase, reduction, or destruction of kappa, as determined by indirect methods, was correlated with the observed number of the stained particles (59).

The question of whether kappa is of intrinsic or external origin is still open, but Preer's failure to find comparable particles in any but killer strains favors the latter interpretation. Altenburg (1, 2) was the first of many to emphasize the possibility that kappa is a symbiont. Many observers report parasites in Ciliophora. Recently, Chen (15) claims that the so-called "chromidia" of Opalinids are parasites; Diller (22) reports a fungoid symbiont in *P. calkinsi;* and Wichterman (103) notes a parasite in *P. bursaria.* However, critical proof of the parasitic nature of these objects has not yet appeared. The symbiotic alga of *P. bursaria* is well-known. These, like kappa, can be reduced or eliminated by rapid growth (42) and by exposure to x-rays (104, 105), and Provasoli *et al.* (60) succeeded in eliminating the green color of *Euglena* by exposure to streptomycin. The close parallels among parasites,

symbionts, plastids, and genetic factors in the cytoplasm have been pointed out and discussed by Sonneborn (85).

GENETICS

RACIAL DIFFERENCES

Possible racial differences in the details of nuclear behavior in *P. trichium* and *caudatum* are mentioned by Diller (21, 22). Nutritional differences distinguishing strains of *Tetrahymena* were reported by Kidder & Dewey (44). Gause (25) and his co-workers found a number of physiological differences among strains of *Paramecium* and *Euplotes* collected in different latitudes. Antigenic differences between races have been noted in the *Tetrahymena-Glaucoma-Colpidium* group by Robertson (63), Tanzer (93), and Kidder *et al.* (45), and in several species of *Paramecium* by Bernheimer & Harrison (7, 8), Harrison & Fowler (32 to 35), Sonneborn (74, 83), and Kimball (47, 48).

Mating Types and the Breeding System in *Paramecium*

For a review of earlier work on genetics of *Paramecium*, see Sonneborn (80). Sonneborn & Dippell (88) added a theoretically interesting observation bearing on the mating type system in *P. aurelia.* This involves a race previously noted as belonging to their variety 4 but differing from the other races of that variety by yielding few or no viable progeny in crosses with the other races. The exceptional race was suspected of representing an early stage in evolution of a distinct variety. Their new observations show that this race is distinguished from other variety 4 races in its mating reactions, for it alone reacts sexually with variety 3. On this basis, the exceptional race was held to belong to a new variety designated as 8. Its sexual reactions permitted extension of the homologies between mating types of different varieties, so that now the homologies can be made for six of the eight varieties. The sexual reaction between varieties 3 and 8 is the first case of mating between varieties of the so-called groups A and B that differ in their genetic systems.

Hiwatashi (36) examined the breeding system among 43 clones of *P. caudatum* collected from 17 natural sources. These clones fall into four noninterbreeding varieties, each with two interbreeding mating types. Five of the 43 clones remain unidentified

and may represent more varieties. The relation of Hiwatashi's four Japanese varieties to Gilman's five American varieties (31) and Y. T. Chen's four Chinese varieties (16) of *P. caudatum* remains unexamined.

KILLER *Paramecium aurelia*

Strains of *P. aurelia* called "killers" liberate into the fluid in which they live a substance, paramecin, that kills paramecia of sensitive strains (Sonneborn, 69, 73). Different killer strains produce different kinds of paramecin that kill in visibly different ways (23, 57, 69). The paramecin liberated by stock 51, variety 4, has been most fully investigated. Austin (3, 4, 5) and others (89, 90, 95) have shown that a sensitive *Paramecium* can be killed by a single particle of this paramecin. Thus under proper conditions the number of sensitive animals killed measures the amount of paramecin present. Using this quantitative method, van Wagtendonk & Zill (97) studied the stability of paramecin at different pH's and temperatures and concluded that its inactivation is a first order reaction with an energy of activation within the range reported for enzymes and proteins. Van Wagtendonk (95, 96) further showed that paramecin is inactivated by pepsin, chymotrypsin, and desoxyribonuclease, indicating that both protein and desoxyribonucleic acid are components essential for its killing action; but it is not inactivated by lysozyme, hyaluronidase, papain, or ribonuclease.

Austin (4) found that killer animals liberate one particle of paramecin every 5 hr. on the average; that the particle may be liberated at any stage of the growth-division cycle; and that the liberated particles settle out. According to Sonneborn *et al.* (90), a few particles of paramecin are liberated when a killer cell is mechanically disrupted and the paramecin may be easily centrifuged down; sensitive paramecia ordinarily take up paramecin quickly, but they are partially or completely protected during conjugation, at low temperatures, and when overfed.

The capacity to produce paramecin, in other words the killer trait, is inherited through the cytoplasm [Sonneborn (73, 77)]. The physical basis of this cytoplasmic inheritance is the kappa discussed above (p. 69). The earlier work of Sonneborn showed that only kappa could make kappa, i.e., kappa is self-reproducing; and this has been beautifully confirmed by Preer's demonstration

(56, 57) that only one, but at least one, kappa particle must be present in a cell in order for kappa to persist and multiply. That kappa can mutate like a gene and then reproduce true to the mutated type was indicated by data of Preer (57), but it remained for Dippell (23) to provide the first full proof of kappa mutability. She analyzed a number of killers which had "mutated" so as to produce different kinds or quantities of paramecin and in every case the change was traceable to a change in kappa.

In a thorough study of some properties of the kappa found in stock G of variety 2, *P. aurelia*, Preer (57) determined the rate of increase of this kind of kappa and the factors influencing that rate. The kappa rate increases with cell fission rate, with the reciprocal of kappa concentration, and with temperature, within certain limits. In variety 2, maximal rate for kappa is about two duplications per day in stock G and less in other stocks; it is less than the maximal fission rate at all temperatures. The number of kappa particles in G killers was determined, by ingenious indirect methods, as about 400 to 500 per cell. Later (58) the x-ray inactivation method indicated 400 to 1,600 particles and eventually (59) direct counts of the stained particles showed about 1,000 particles. Preer (57) points out the general consequences of amitotic distribution of self-duplicating cytoplasmic particles like kappa, particularly the necessity for compensatory mechanisms to prevent excessive accumulation or depletion.

The presence of kappa in a cell is not enough to assure paramecin production. When kappa is present in low concentrations, the cells not only fail to produce paramecin but are killed by paramecin (77); indeed there is a graded series of phenotypes from sensitive to resistant to weak killer to strong killer in dependence upon concentration of kappa in the cell (56, 57). The concentration of kappa in a cell may be varied in a number of ways. It may be quantitatively destroyed by exposure to x-rays [Preer (58)], high temperature [Sonneborn (79)], nitrogen mustard gas [Geckler (26)]. It may be reduced by growing the paramecia faster than kappa can multiply and it may be increased by adjusting conditions to reverse these relative rates [Preer (56, 57)]. Kappa may be introduced into cells that previously lacked it by establishing cytoplasmic bridges with killers during conjugation [Sonneborn (73, 77)] and by exposing sensitives to high concentrations of

kappa (10^8 particles per cc.) obtained by crushing concentrated cultures of killers [Sonneborn (82)].

The question of the relation of kappa to paramecin remains unanswered (82). Both contain desoxyribonucleic acid (58, 59, 95, 96); both are present in the bodies of killer animals (90); both are unstable and are inactivated at rates varying with temperature (89, 95, 96); both are highly active, single particles being decisive (4, 5, 56, 57). Yet there seem to be important differences. Determinations of particle numbers in killers yield high values for kappa (56 to 59, 79), low values for paramecin (90). When sensitive animals of proper constitution receive a single particle of paramecin, they die (4); but when they receive a single particle of kappa the kappa multiplies and converts them into killers (56, 57, 82). Evidence for multiplication of paramecin, even in sensitive animals, has thus far been impossible to obtain (82).

Although kappa has the fundamental genic attributes of self-duplication and mutability the question of whether it should be considered a plasmagene or parasite (see p. 69 above) is difficult; for particles like kappa, the question is perhaps academic (85).

The maintenance and multiplication of kappa depend upon the genetic constitution of the paramecia. In variety 4, the gene *K* must be present (73); when gene *S* is also present another cytoplasmic factor, sigma, controlled and possibly initiated by gene *S*, may compete with kappa and lead to the disappearance of the latter (81).

Antigenic Traits in *P. aurelia*

Bernheimer & Harrison (7, 8) called attention to the existence of many antigenically different stocks of *P. aurelia*. Harrison & Fowler (33) obtained hereditary antigenic variants within a stock. Although most of these were isolated following exposure to paralyzing antiserum, some arose spontaneously. They therefore concluded that all were spontaneous variants, the antiserum serving merely to select them. Sonneborn (80) interpreted their data as indicating the existence of more than one type of variation.

That exposure of paramecia to antiserum could result in persistent resistance to it was indicated (65) long before Jollos (43) established this fact. It has subsequently been confirmed by Sonneborn (75, 81) and by Kimball (47). Inheritance of induced

resistance to antiserum follows a different course in different varieties of *P. aurelia*. In Jollos' material, resistance persisted for variable periods of reproduction by fission, but disappeared at the first fertilization. Similar results were obtained on group A variety 1 of *P. aurelia* by Sonneborn (75) and Kimball (47). In group B variety 4, on the other hand, the resistance persists without limit through successive fertilizations [Sonneborn (81)]. Clearly, the work on the two kinds of material must be separately considered.

Kimball's work (47, 48) on variety 1 showed that resistance could be induced also by exposure to trypsin or by growth at 14°C. There was great variation in the number of fissions through which the acquired resistance persisted at 28°C., but the number was in many cases so large as to convince Kimball that the antigens or some precursors of them are self-duplicating or in some other way facilitate the production of more of themselves. The transforming conditions are held to operate by inactivating antigen (by means of antiserum and trypsin) or by shifting the equilibrium concentration of antigen (by means of low temperature) through a disturbance of the relative rates of antigen production and cell multiplication.

In Sonneborn's work (81, 83) on variety 4, it was shown that resistance to antiserum is accompanied invariably by acquirement of sensitivity to another antiserum; in other words, decrease or loss of certain antigens is accompanied by corresponding gain or increase of others. At least six alternative antigenic types can exist in each of the two stocks examined: types A, B, C, D, E, and G in stock 51; types A, B, C, D, F, and H in stock 29. The various antigens characterizing these types seem to be mutually exclusive, so that only one is manifested in any one line of descent.

Beale (6) analyzed in detail the transformation of type D to type B following exposure to anti-D serum. Immediately after exposure, the animals are still type D and during the following 15 to 27 hr. the reaction to anti-D decreases while reaction to anti-B increases. Active metabolism is involved in the transformation, for prolonged starvation inhibits it.

In the early work (81, 91) on variety 4, study of cross-reactions and absorption led to the view that all antigens producible in a stock were present in animals of every antigenic type, but in different proportions. One antigen was present in large amount, but the others were believed to be present in small amounts.

Transformation thus seemed to involve shifts in concentration of preexistent antigens. Further work (87) reveals only one paralytic antigen in each antigenic type. Transformation thus seems to involve loss of one antigen and its replacement by another one not previously detectable, but in the spectrum of possibilities characteristic of the stock. The cross-reactions of the various sera may be due to a fundamental close similarity among all the alternative antigens and to variation in antibody production by the rabbit when injected with a single one of these antigens.

Transformation of antigenic type is readily induced in up to 100 per cent of the animals by various means. Exposure to specific paralyzing antiserum is highly effective (81, 83, 91), but alone does not determine which new type will arise. Control of direction of transformation can be obtained by varying temperature and food supply during the first day or two after exposure to antiserum (83). These determinative conditions (temperature and amount of food) can bring about transformations without preliminary use of antiserum, but the transformations do not take place until relatively long periods (10 to 30 fissions) have elapsed under these conditions (83). At 27°C. with food enough for only one fission per day, all types are more stable, some almost absolutely, others much less so. But the changed types are reversible under appropriate transforming conditions (83).

The various antigenic types, regardless of whether they arise spontaneously or under transforming conditions, are thus hereditary under standard conditions. Breeding experiments show that within a stock the diverse types are identical in genes and that inheritance follows the cytoplasm (81, 83, 91). However, differences in the antigenic possibilities that distinguish different stocks are due to gene differences (83). The genes are apparently capable of initiating production of the cytoplasmic basis of the antigenic types (83).

The nature of the cytoplasmic factors involved in control of antigenic types is still unsettled. Much of the analysis implied a system of competing plasmagenes (83, 91) and the antigens themselves seemed to be the plasmagenes [Beale (6)]. Delbrück (18) has, however, suggested a scheme of reciprocally inhibitory reactions which can account for cytoplasmic inheritance without resort to plasmagenes. Experimental distinction between these alternative views is difficult to obtain. However, reasoning that

plasmagenes, if they exist in this case, should show the property of mutability, Sonneborn (86) reports an indirect test of the capacity of the cytoplasmic basis of antigenic type to mutate and the results were negative. The work of Sonneborn and his associates on inheritance of antigens in *P. aurelia* has been reviewed in part elsewhere (84, 85, 86).

EVOLUTION

Gause (25) presents in English a review of evolutionary studies on *Paramecium* and *Euplotes* that have been published mainly in Russian. The theme of these studies is the concept of organic, stabilizing, or coincident selection long ago set forth by Baldwin, Osborn, and Lloyd Morgan. According to this concept, natural selection preserves mutations which alter an organism in the same direction as its response to modifying environmental conditions. This leads to a pseudo-Lamarckian result by means of orthodox genetic mechanisms.

Vegetative animals of *Euplotes vannus* cannot survive in 7 per cent saline, but 5 per cent saline results in a phenotypic decrease in body size. On the other hand, a population of conjugants includes some that survive 7 per cent saline and in 5 per cent saline the average decrease in body size is greater than for vegetative individuals. Moreover, in 1 per cent saline, the body size is increased in exconjugant populations, but not in the parent clones. From these observations, it is concluded that, under conditions which do not elicit an adaptive response (change of body size), adaptive genotypic variations are selected; and that, under conditions which do elicit this adaptive response, genotypic variation reinforces it.

The frequency of adapted exconjugants in 7 per cent saline is about 1 in 1,200 and this is held to indicate recombination involving at least 5 or 6 gene loci. However, no proof is given that any of these variations is genotypic. The need for such proof is emphasized by the fact [see (80)] that entirely homozygous cultures of *Paramecium* give rise to hereditary variations of unknown type. Jollos called such variations Dauermodifikationen and showed that they were impermanent but long lasting changes induced by environmental conditions. He further believed the organisms were particularly sensitive to such conditions during and immediately after fertilization. The results of Gause and his

collaborators on *Euplotes* cannot be securely interpreted without distinguishing experimentally between the alternative interpretations of gene recombinations and Dauermodifikationen.

The same investigators have analyzed the reactions of *Paramecium caudatum* to saline and to quinine. Here a distinction is made between initial adaptability of the organism and further adaptation developed slowly (acquired adaptation). It is claimed that clones with small initial adaptibility have correspondingly larger capacity for acquired adaptation. This is believed to indicate a compensation between initial and acquired properties. Adaptive modification and genoadaptation in microevolution are considered to be equivalent to plasticity and specialization in long-range evolution.

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METABOLISM OF MICROORGANISMS

By W. W. Umbreit

Merck Institute for Therapeutic Research, Rahway, New Jersey

The subject of microbial metabolism draws its information and its methods from what normally would be separated disciplines. What one observer regards as significant may not satisfy another, hence the present review is not to be regarded as a complete survey of publications of the past year, but is an attempt to survey certain areas of the subject currently of importance. This review covers the period from December, 1947 to December, 1948, with the addition of a few further papers appearing in early 1949 whose mention was judged necessary to complete the discussion of some aspects of metabolism.

The past year evidences a marked increase in the successful exploration of those few methods which are available for the study of pathways of biological syntheses and contains increasing reports on the locus of action of antibiotics. Two books, of interest to every student of microbial metabolism, have appeared. First, Glick (1) has provided an excellent description of histological, chemical, and manometric microtechniques. These are clearly presented and should prove most useful especially to those engaged in the study of reactions occurring below the range of the more usual methods. Second, Work & Work (2) in a book entitled *The Basis of Chemotherapy* have presented remarkably complete discussions of bacterial metabolism, essential metabolites and their functions, enzyme inhibitions and drug antagonism, particularly antagonisms of essential metabolites. Almost 60 pages are devoted to a discussion of drug resistance from the metabolic viewpoint.

Carbohydrate Metabolism

The usual view of carbohydrate metabolism holds that saccharides are broken down by way of a common intermediate system to pyruvate, which then follows a variety of alternative pathways dependent upon the organism and the conditions. Other substances enter into these metabolic pathways at various points. The concept of a "basic metabolic ground plan" has suffered no severe blows in the studies of the past year. A general discussion of the

metabolism of molds was given by Foster (3) and an excellent summary of what little is really known of the metabolism of the thermophilic organisms by Gaughran (4). A portion of work on metabolism of the malarial parasites, from which other data may be found, has been presented (5) and will not be further discussed except to note that apparently there is little correlation between antimalarial activity and respiratory inhibition (6). There seems to be agreement (7, 8) that respiration of microorganisms is little influenced by the presence of immune sera providing lysis does not occur (8). McIlwain (9) has employed abrasive grade alumina for grinding bacterial cells and has thus obtained the glycolytic system, arginine dihydrolase, and a nucleotide deaminase in the cell-free state from cocci. The metabolism of bacteria absorbed in thin layers on solid adsorbents, growing out of the somewhat novel approach to soil bacteriology employed by Quastel and co-workers, deserves further attention (10). There is further data on the formation and properties of various polysaccharides found in bacteria (11, 12, 13). The bacterial production of amylase has been described (14, 15, 16).

With respect to the fermentation of disaccharides themselves, lactase has been isolated from *Escherichia coli* (17). Galactose fermentation by yeasts has been studied from the point of view of adaptive enzyme formation (18, 19). While a particular genetic background is necessary before galactase formation is possible, a cell possessing that background does not necessarily have the ability to ferment galactose. The presence of galactose and a sufficient source of energy are necessary before the galactose fermenting enzyme is formed. Anaerobically, galactose itself will not serve as such an energy source since it cannot be fermented in the non-adapted cell; aerobically, however, it can be oxidized and the energy so derived employed to synthesize the galactose fermenting enzyme. One would be curious about the mechanism involved in the oxidation of galactose by cells which do not ferment it. A phosphogalactokinase has been obtained from *Saccharomyces fragilis* (20) catalyzing the reaction of galactose and adenosinetriphosphate to form galactose-1-phosphate. It was further reported (21) that a phosphoglucomutase preparation, capable of converting galactose-1-phosphate to a reducing ester, and glucose-1-phosphate to glucose-6-phosphate, required a heat stable unidentified factor for its activity.

Elsewhere it has been shown that adaptation (of *Aerobacter aerogenes*) to glycerol oxidation does not result in increased ability to dehydrogenate other polyhydric alcohols nor does adaptation to sucrose give increased oxidation of other di- or polysaccharides (22). However, one may point out that such simultaneous adaptation would be expected only to a reaction chain and not necessarily to a similar compound.

Fales & Baumberger (23) report on anaerobic assimilation by yeasts and Swanson & Clifton (24) report a similar study under aerobic conditions. Knox *et al.* (25) place the principal action of chlorine as an antiseptic on inhibition of the glycolytic system, since inhibition of the bacterial aldolase is paralleled by per cent bacteria killed. Gest & Kamen (26) report on the phosphorylated intermediates of fermentation and oxidation in algae, photosynthetic bacteria, and yeast. It is rather difficult to reach any conclusions from the data presented since, as has been the experience of previous investigators, present methods are not adequate for accurately determining the distribution of phosphorus in these tissues, perhaps because of the occurrence of polyphosphates in them. The present studies do not invalidate in the least, as the authors suggest, data from other tissues for which adequate methods have been developed.

An extensive study on the turnover and distribution of phosphate compounds in yeasts during the fermentation of glucose in the presence and absence of exogenous nitrogen has been reported by Spiegelman and associates (27, 28). At least four groups of phosphate compounds not normally encountered in animal tissues were found. These were inorganic metaphosphate, which was acid soluble, a pyrophosphate associated with the phosphoprotein, a labile phosphate (perhaps ribose phosphate) associated with the desoxyribonucleic acid, and a metaphosphate fraction apparently bound to polysaccharide. The kinetics of turnover of the materials are too complex for adequate interpretation, although an attempt has been made to do so. Using similar techniques, it has been demonstrated (28) that relatively low concentrations of azide, which do not interfere with the rate of breakdown of glucose, markedly inhibit the ability of the cell to esterify inorganic phosphate. On the basis of several types of data presented it is suggested that azide reacts with the acyl phosphate of diphosphoglycerate and the resulting acyl azide, being spontaneously decomposed, acts

as a drain on the energy-rich phosphate formed. This concept can very possibly be generalized to cover those agents which "uncouple" phosphorylation (and inhibit oxidative or fermentative assimilation); i.e., they act not by preventing the formation of the energy-rich phosphate but by reacting with it after formation and directing the energy to useless channels. An earlier paper by Gottschalk (29) analyzes the effects of addition of reducible substances to yeast fermentations pointing out that in the strains used, the carrier system from dihydrocozymase to oxygen was relatively weak which thereby permitted other reducible materials to complete successfully with oxygen for this hydrogen.

The anaerobic end products of fermentations are still a matter of considerable study but apparently no longer occupy such a large portion of bacterial metabolism studies. *Clostridium kluyveri* (30) requires ethanol and acetic acid (replaced by propionate and, to some extent, butyrate) for its growth, forming therefrom butyric and caproic acids. Tracing these reaction mechanisms further (31) it was found that when ethanol was limiting and acetate in excess, butyrate was the principle product; when acetate was limiting and ethanol in excess, caproate was formed. Acetate is converted into butyrate and the latter into caproate but the mechanism is still speculative. *C. lactoacetophilum* in pure culture converts lactate to acetate and the latter to butyrate (32). Contrary to the literature and the belief of the past years, it has now been claimed (33) that washed suspensions of *C. saccharbutyricum* and *C. acetobutylicum* will ferment β-hydroxy butyrate (to acetate and butyrate) and that β-hydroxy butyrate is an intermediate in the formation of butyrate by these organisms. Reports on production of butylene glycol by *Aerobacter aerogenes* (34) and *Serretia* sp. (35) will not be discussed.

During fermentations two carbon compounds are produced. These eventually appear as acetate and the metabolism of this compound is the subject of much active research. The reversibility of the phosphorylative reaction producing hydrogen from pyruvate in *C. butylicum*, in which formate is not an intermediate, has been demonstrated (36). *C. kluyveri* can form acetyl phosphate from acetaldehyde and from acetoacetic acid (37).

The microbial synthesis of acetylcholine (38) (by strains of *Lactobacillus plantarum*) has been studied further and pantothenic acid has been shown to be involved (38, 39). The latter has been

further shown to be involved in the oxidation of acetate by yeasts (40). The relation of acetyl phosphate to these reactions is summarized by Kaplan & Lipmann (41) in a short note which demonstrates that the product of the reaction of adenosinetriphosphate (ATP) and acetate is not acetyl or diacetyl phosphate (but may be converted into acetyl phosphate at pH 1.5) and that the activity of acetyl phosphate under some circumstances is probably due to the latter's ability to form "reactive acyl phosphate" by phosphorylation of adenosinedisphosphate (ADP) to ATP. As is well known, pantothenate (in the form of coenzyme A) is involved in the formation and perhaps the activity of the reactive acetyl. McElroy & Dorfman (42) report that *Proteus morganii*, grown deficient in pantothenate, converts a large part of pyruvate supplied to acetoin; when supplied with pantothenate, more oxygen is taken up and little acetoin is formed. This is interpreted to mean that pantothenate is concerned with the oxidation of acetoin. However, the more likely alternative is that in the deficient cell, oxidation of pyruvate to acetate and beyond is diminished so that pyruvate takes the alternative route to acetoin. An unidentified factor, not pantothenate or coenzyme A, is required for the oxidation of pyruvate by certain enterococci (43).

The mechanism of acetate oxidation is now an active and controversial field. At the present writing there appear to be four alternatives: (*a*) acetate is condensed with oxaloacetate and oxidized via a tricarboxylic acid cycle; (*b*) acetate condenses with itself or with pyruvate to form acetoacetate which is oxidized; or (*c*) to form succinate which is oxidized. Finally (*d*), acetate is oxidized by way of the methyl carbon. The situation is such, however, that clear proof of the existence of even one of these mechanisms is still lacking. This is not solely due to different organisms employing different mechanisms, but to the actual lack of unequivocal data on even one organism. In yeast the oxidation of acetate traced by C^{13} (carboxyl labeled) led to citrate and carbon dioxide and seemed to support the concept of the tricarboxylic acid cycle (44) (the tracer was also found in the lipid fraction) while Lynen (45) reports that acetate does not go through the citrate stage. By means of traced acetate it is demonstrated that over half of the glucose oxidized by yeast goes through the acetate stage (46). Acetate oxidation is inhibited by fluoroacetate (47) but this does not provide evidence of mechanism. Karlsson & Barker (48, 49), studying acetate oxi-

dation in *Azotobacter agilis*, have provided evidence showing that the complete tricarboxylic acid cycle does not exist in this organism. The data show that the portion apparently missing is the pyruvate-oxaloacetate condensation and that the remainder is adaptive. There is, incidentally, rather good evidence that α-ketoglutarate is oxidized by way of succinate, fumarate, malate, and pyruvate. Yet acetate is oxidized to completion and is not, therefore, oxidized by way of the tricarboxylic acid cycle. No alternative mechanism was found. The data would also apparently eliminate the condensation of acetate to succinate.

Related to these problems is the formation of fumarate by *Rhizopus* in which it has been proposed (50) that there exist two mechanisms of fumarate formation, a C_3+C_1 condensation occurring primarily under anaerobic conditions and a C_2+C_2 condensation occurring aerobically. The fixation of carbon dioxide in α-ketoglutarate by a cell free enzyme from *E. coli* has been reported (51). This would be an example of a C_4+C_1 condensation. *Lactobacillus arabinosus* when grown with malate is capable of converting it largely to lactate and carbon dioxide (52) and might prove to be a useful tool in the study of the malic dehydrogenase system. Johns (53) in a brief note reports the formation of propionate by decarboxylation of succinate in an organism isolated from sheep rumen. Further, Delwiche (54) reports a study of succinate decarboxylation in *Propionibacterium* sp. in which it is shown that the rate of decarboxylation is sufficient to account for the rate of formation of propionate. In addition propionate, acetate, carbon dioxide, and hydrogen have been reported in the fermentation of lactate by a micrococcus (55). An organism which will grow on oxalate and oxidize formate, oxalate, and pyruvate has been reported (56). The preparation of some of the enzymes concerned in glycerol oxidation by *Staphylococcus aureus* has been described (57). Several papers report the chemical properties of the products of the oxidation of various cyclotols by *Acetobacter* sp. (58 to 61) in one of which (58) it is demonstrated that *Acetobacter suboxydans* is capable of oxidizing only those hydroxyls which are situated in a polar plane. The oxidation mechanisms of attack on aromatic compounds (62, 63) and steroids (64) have been studied.

Nitrogen Metabolism

Bacterial amino acid decarboxylases have been the subject of

a surprising number of papers during the past year. Blaschko & Stanley (65) have shown, in a short report, that the bacterial tyrosine decarboxylase requires hydroxyl substitution in the para position of phenylalanine, whereas the comparable enzyme ("dopa decarboxylase") from guinea pig kidney requires meta substitution. Thus, while both enzymes will attack dopa (*m*-, *p*-, dihydroxyphenylalanine), the bacterial enzyme will attack only tyrosine as well. This would be all very well except that McGilvery & Cohen (66) have shown that the bacterial tyrosine decarboxylase preparations also decarboxylate phenylalanine, which possess no hydroxyl substitutions; and there is as yet no reason to suspect that an additional enzyme is involved. This observation the reviewer has confirmed and can add that pyridoxal phosphate is also the coenzyme of the phenylalanine decarboxylation. The aspartic decarboxylase seems to be a very weak enzyme and rarely occurs in sufficient quantity to measure by carbon dioxide evolution [except possibly in a "pseudo mycobacterium" (67, 68, 69)], but it has been prepared from *E. coli* in a cell free state (70). Histidine dipeptides are apparently attacked by histidine decarboxylase to yield amine substituted histamines (71) and histidine decarboxylase has been studied further (72). A general review of the decarboxylase enzymes has also appeared (73).

Acetate is not an intermediate in the formation of butyrate from threonine (74) by *C. propionicum*, but here it would seem probable that a threonine deaminase yielding α-ketobutyrate with its subsequent reduction might well be involved in the origin of the butyrate. In *Diplococcus glycinophilus*, which ferments glycine to form ammonia, acetate, and carbon dioxide, it was shown (75) that most of the acetate arises from the methylene group of the glycine and most of the carbon dioxide from the carbonyl group, from which it was postulated that two glycines condense through the methylene carbons presumably forming an α-β-diaminosuccinate which was then decarboxylated, deaminated, and oxidized so that the two central carbons contributed the acetate. Other deaminations have been studied. The L-amino acid oxidase of *Penicillium notatum* was obtained (76) although compared to enzymes from animal tissues and bacteria its activity seems rather low. Dawes (77) has summarized the very extensive literature on the enzyme in *E. coli* which produces indole from tryptophane, which generations of bacteriologists have called tryptophanase, although Dawes

now wishes to apply this term to a more complex system.

The aspartic deaminase is being used as a tool for the study of biotin function. Lichstein & Christman (78) report that this enzyme (and the serine and threonine deaminases) responds to biotin and to adenylic acid in such a manner as to suggest that adenylic acid is concerned with the formation of a biotin containing coenzyme. Axelrod *et al.* (79) claimed that biotin was not concerned in the activity of these enzymes, but reports appearing in early 1949 (80) provide further evidence of the existence of biotin in a coenzyme form and offer an explanation (81) for the results of Axelrod *et al.* The relation of biotin to yeast fermentation has been reported (82). Biotin-deficient and oxybiotin-deficient yeast have a lower fermentation rate which can be stimulated by the addition (in the presence of ammonia) of biotin or oxybiotin, and by aspartic acid. The stimulation due to the biotin compounds could be antagonized by biotin analogues; that due to aspartic could not. The analogues were capable of inhibiting the biotin or oxybiotin effect only when added prior to the vitamin. These data could readily be interpreted as an involvement of biotin in aspartic acid synthesis in a coenzyme or "bound" form but this still leaves one with the effect of aspartate in stimulating glycolysis for which a reasonable explanation is lacking.

The pathway of synthesis for various amino acids and related compounds is slowly being clarified. It has been shown that in *Neurospora*, cysteine goes to homocysteine by way of cystathionine (83), and that homoserine goes to threonine and methionine (84). By means of inhibition analysis a relationship between methionine and leucine has been indicated (85). By means of growth studies anthranilic acid is indicated as the precursor for indole (and tryptophane) in *Eberthella typhosum* (86) and it accumulates in the presence of 4- and 5-methyl-anthranilates. The enzyme catalyzing the indole-serine condensation to tryptophane is absent in the *Neurospora* variant which can grow on tryptophane but not on indole (87). In *Neurospora*, it has further been shown that the precursor of lysine is α-amino adipic acid and not α-keto adipic acid or α-α diamino adipic acid (88). In *Neurospora*, hydroxyanthranilic acid is the precursor of nicotinic acid (89). Some clue to the interrelationship, in fact to the synthesis, of anthranilic acid, indole, tryptophane, and nicotinic acid may be offered in a brief report (90) that *E. coli* incubated with ammonium lactate and a

variety of other materials synthesized nicotinamide in quantity only when ornithine was added.

Pyridoxal phosphate does not function in transamination by reversible conversion to the corresponding amine (91). Considerably more data on glutamine synthesis has been presented (92, 93) and a relationship between proline and streptomycin has been reported (94). Studies of purine, pyrimidine, and nucleic acid synthesis are advancing rapidly. Glycine has been shown to be a precursor of purines in yeast (95), its nitrogen entering at the 7 position. 5(4)-Amino-4(5)-imidazolecarboxamide is a precursor of purines which accumulates under conditions of sulfonamide inhibition (96). Glycine is involved in the synthesis of the carboxamide and possibly threonine. Folic acid is thought to be involved in the addition of a single carbon unit into the structure of purines, pyrimidines, and probably histidine (97). In lactobacilli, on a low pteroylglutamate medium, less desoxyribose nucleic acid was found (98). When thymine was added the level of the desoxynucleic acid increased. The synthesis of folic acid by resting cells from glucose, glutamate, and *p*-aminobenzoate has been studied (99).

In the synthesis of pyrimidines some evidence is available that the carbon chain of oxaloacetate appears in the orotic acid involved in the formation of uracil (100). Incidental to describing a chromatographic method for pyrimidines, Chargaff & Kream (101) demonstrated a cystosine deaminase in *E. coli*.

Nucleic acid metabolism in microorganisms is being intensively studied. Work which may well be of historic importance in the field of nucleic acid synthesis in relation to bacteriophage and virus formation has appeared within the past year (102 to 113). While there are many points of difference in the papers noted, the detailed discussion of which is more appropriate elsewhere in this volume, two things seem to be common to all the systems studied. Apparently the major portion of material which goes into the formation of the virus is derived not from performed materials in the cell but is synthesized more or less directly from the constituents of the medium. Infection with the virus causes an increased absorption of nutrient which is directed almost exclusively toward the synthesis of the virus. Further, several systems have been obtained in which phage formation could be demonstrated without bacterial growth and a further extension of these techniques may

actually permit bacteriophage formation in a cell free state. Even before this has been accomplished a great deal can be learned of the pathway of nucleoprotein synthesis.

A very curious reaction has been reported (114) in which it was shown that a gram negative organism (*E. coli*) could be made to yield a gram positive stain by treatment with viscous desoxyribonucleic acid.

Action of Antibiotics

Penicillin.—A discussion of the mode of action of penicillin may be based on the studies of Gale and co-workers (115 to 119) on glutamic acid assimilation in gram positive bacteria. Glutamic acid enters the cell as a result of an active process utilizing energy and undergoes a marked concentration. The level found within the cell depends upon the rate at which glutamate enters the cell and the rate at which it is metabolized by the cell. The internal metabolism of glutamate may be roughly divided into two large categories (116): some of the glutamate is condensed into protein (this process is inhibited by sulfa drugs); another portion enters into other metabolic reactions whereby its character as glutamate is lost [glutamine formation was studied, this reaction was inhibited by crystal violet (117)]. Penicillin was demonstrated (118) to interfere with the absorption of glutamate from the external environment but not to interfere with the internal metabolism of glutamate once it was inside of the cell. However, penicillin does not prevent the assimilation of glutamate in washed suspensions of cells grown in the absence of penicillin (119). This blocking of glutamate assimilation occurs only if the cells are grown for a short period in the presence of penicillin. Other properties of the cells (except viability) such as glucose oxidation or fermentation are quite unaffected by growth in penicillin. Since the process of glutamate absorption from the external environment occurs in the presence of penicillin so long as the cells do not grow or have not been grown in penicillin, the latter's action must not be on the absorption of glutamate per se but upon the formation of the enzymes necessary to absorb the glutamate. These are so far unknown (and, of course, cannot be prepared cell-free since they are characterized by the ability to form a concentration gradient) but some rather important advances can still be made. The gram negative cells, while they certainly absorb amino acids, are apparently

unable to carry out an internal concentration of the amino acids. Perhaps, therefore, it is the actual substance involved in the gram stain which is also involved in the internal concentration of certain amino acids. Therefore, the effect of penicillin upon the synthesis of nucleic acid by growing cultures was studied with the result that in the presence of penicillin the ribonucleic acid decreased (per mg. dry weight of cells), while desoxyribose nucleic acid actually increased compared to cultures grown in the absence of penicillin. The penicillin therefore somehow interferes with the formation (or stimulates the breakdown) of the ribonucleic acids, but until more is known of the mechanisms of these processes the exact locus of action cannot be specified.

In pursuing the penicillin problem further, resistant strains were derived and studied by two separate laboratories (119, 120) from which it may be concluded that there is a relationship betweeen synthetic abilities and penicillin resistance. When resistance is pushed to very high levels organisms result which can synthesize most if not all of their amino acid requirements (and such organisms tend to be gram negative). Reversion of these to penicillin sensitivity (and a gram positive character) results in strains with wider amino acids requirements. Other metabolic changes may occur, as for example that reported by Bellamy & Klimek (121) in which a resistant strain of *S. aureus* had lost the ability to grow anaerobically. Nevertheless, the nutritive requirements, penicillin resistance, and gram staining character can now be correlated in a manner hitherto impossible and which would seem to bear directly upon the role of penicillin in inhibiting the absorption of certain amino acids which seems to arise from its action in inhibiting nucleoprotein synthesis. Why penicillin is able to enter the animal body without harm to the host is yet to be experimentally approached.

Streptomycin.—The report of Fitzgerald & Bernheim (122) that streptomycin inhibited the oxidation of benzoic acid in acid-fast organisms was followed by data (123, 124) showing that streptomycin was preventing the formation of the adaptive enzyme concerned in benzoate oxidation, hence its action in inhibiting the growth of these organisms must be due to some other reaction. The effect of urea was traced to the formation of cyanate which chemically inactivated the streptomycin (125). While perhaps not related to streptomycin, it is curious that sodium fluoride should

increase the respiration of the mycobacteria (126). Benham (127) reported that 1,000 units of streptomycin per ml. increased the oxygen uptake of *Eberthella typhosa*. A more extensive study by Henry *et al.* (128) showed that streptomycin did not inhibit a variety of enzymes even at very high concentrations, yet bacteriostatic concentrations did inhibit the carbohydrate metabolism of susceptible strains while having little effect upon resistant strains. When inhibited, the carbohydrate fermentations were characterized by the accumulation of acetate. These results are entirely compatible with the notions of mechanism current in the reviewer's laboratory, as published in early 1949 (129). Based upon the work of Geiger (130) in which an effect of streptomycin on the oxidation of amino acids (following a preliminary period of carbohydrate oxidation) was described, it could be demonstrated (129) that the inhibition was concerned with the terminal respiration of the keto acids derived from the amino acids and that streptomycin specifically inhibited the oxaloacetate-pyruvate condensation or a reaction very close to it. Such a locus of action would lead to the accumulation of acetate in the inhibited cells as reported by Henry *et al.* (128).

Other drugs.—The pyruvate oxidase of *Proteus vulgaris* is inhibited in the intact cell by protoanemonim but when the enzyme is prepared cell-free, little inhibition is evident (131). The dehydrogenase activities on several substrates of cells of pneumococcus rendered resistant to several drugs tended to be lower than the parent susceptible strains (132), but what activity remained was not as sensitive to the presence of the drug as was the comparable reaction in the susceptible cells (133). Cell free extracts (134) showed the same phenomenon hence it seemed probable that the actual enzyme proteins had been altered upon acquiring resistance. Synthesis of *Lactobacillus casei* factor (folic acid) by resting cell suspensions of *Streptobacterium plantarum* was inhibited by sulfonamides and the inhibition was overcome competitively by *p*-aminobenzoate (99).

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NUTRITION OF MICROORGANISMS[1]

By Esmond E. Snell
Department of Biochemistry, University of Wisconsin
Madison, Wisconsin

Space limitations and the special interests of the author preclude an exhaustive presentation of advances in knowledge of the nutrition of microorganisms. Instead only special topics which have been clarified or advanced during the past year will be treated. The very large literature which has accumulated on the use of microorganisms (especially the lactic acid bacteria) for the quantitative determination of amino acids will not be summarized. This field has been reviewed at fairly frequent intervals (1, 2, 3). Several books (4, 5, 6) or reviews (7) dealing with the application of microorganisms to the determination of vitamins have also appeared, and only infrequent reference to such procedures will be made below.

The most active field of nutritional research in microbiology remains that of attempting to identify the organic compounds required for growth of individual organisms in defined media. Such compounds may serve as sources of energy, or as essential structural or catalytic components of protoplasm. If the fundamental postulate of an essential similarity in constitution of functional protoplasm from whatever source (modified, to be sure, by environmental conditions and all the multiple influences of a divergent evolution toward dissimilarity) be accepted, the fact that the same amino acids and vitamins continually recur in different numbers and different combinations as growth essentials for the most diverse organisms should not occasion surprise. Examination of any exhaustive review (e.g. 8, 9) of the nutritional requirements of microorganisms demonstrates that this is the case. The view that these requirements arise through loss of the capacity to synthesize substances of general importance in metabolism (cf. 10, 11) has been underscored and given a genetic basis by the induction of biochemical mutants in fungi and in bacteria which require these same substances for growth 12, 13, 14).

Accordingly, one might expect eventually to find, either in natural populations or induced mutants, a nutritional requirement

[1] This review covers the period from March, 1948 to March, 1949.

for almost every diffusible catalyst or structural unit of fundamental importance to metabolic processes. While there are adequate reasons for suspecting that this will never be fully achieved, the point of view serves to emphasize the important role which research on unidentified growth factors may play in elucidation of fundamental biochemical materials and processes.

NEWLY IDENTIFIED GROWTH FACTORS

Vitamin B_{12}.—In 1947 Shorb (15) reported that *Lactobacillus lactis* Dorner required two unidentified substances for growth in a medium containing the known vitamins and amino acids. One of these, designated the TJ factor, was supplied by clarified tomato juice, and its nature remains unknown. The other, designated as LLD factor, was present in high amounts in refined liver extracts of the type used in treatment of pernicious anemia. Guided by this assay method, Rickes *et al.* (16) isolated from such liver extracts a crystalline red compound which was active as the LLD factor and supported half-maximum growth of *L. lactis* at a concentration of approximately 0.013 mμg. per ml. of culture medium (16, 17). The same compound proved to be active at extremely low dosages in the cure of pernicious anemia in man (18) and has been tentatively called "vitamin B_{12}." Although the chemical structure of this substance is not yet known, it appears to be a coordination complex of cobalt and contains cobalt and phosphorous in the ratio of 1:1. It has a minimum molecular weight (based upon its cobalt content) of approximately 1,500 (19, 20). It is labile to alkali and acid at room temperatures but is not destroyed by autoclaving near neutrality at 15 lbs. pressure for 15 min. Cobaltous ion does not replace the vitamin in the nutrition of *L. lactis* (20).

Much difficulty has been encountered by those attempting to utilize *L. lactis* for assay of vitamin B_{12}. Shorb & Briggs (21) indicate that dissociation of the culture occurs in some media, giving rise to variants which do not require vitamin B_{12} for growth. Perhaps a more common cause for trouble is the nonspecificity of the response to vitamin B_{12}, discussed below.

Many other lactic acid bacteria require vitamin B_{12} under appropriate conditions. These include two strains of *L. leichmannii* (22, 23), *L. bifidis* (24), and several strains of *L. acidophilus* (25). Among the protozoa, *Euglena gracilis* var. *bacillaris* has been shown to require the crystalline antipernicious anemia factor

(vitamin B_{12}) for growth (26). Thus this vitamin has been shown to be of importance in organisms as diverse as bacteria, protozoa, and man. Although little is yet known of its distribution, many bacteria, including *Escherichia coli*, *L. casei*, *Streptococcus faecalis* (21), *L. arabinosus*, *Mycobacterium smegmatis*, *Bacillus subtilis*, and several species of *Streptomyces* (27), are reported to synthesize it. The crystalline vitamin, identical with that from liver, has been isolated from a grisein-producing strain of *Streptomyces griseus* (27).

Thymidine, other desoxyribosides, and ascorbic acid, and their relationship to vitamin B_{12}.—Thymidine was first implicated in bacterial nutrition by Shive and co-workers (28), who isolated it from liver extracts and showed that it overcame the inhibitory action of methylfolic acid for *Leuconostoc mesenteroides* in a medium that contained thymine. Shortly thereafter, Shive, Ravel & Eakin (29), and Wright, Skeggs & Huff (30) independently showed that this compound replaced vitamin B_{12} in the nutrition of *L. lactis* and Snell, Kitay & McNutt (31) showed that it replaced crude natural extracts in promoting growth of several strains of *L. acidophilus*, one strain of *Leuconostoc citrovorum* and two of *L. leichmannii*. The latter two organisms have since been shown to grow with vitamin B_{12} replacing thymidine in the medium (22, 23). Shive *et al.* (29) had further shown that addition of ascorbic acid to the medium also rendered vitamin B_{12} (or thymidine) nonessential for *L. lactis*. Further investigation by Kitay, McNutt & Snell (32) showed that individual addition of any one of the following seven substances to an otherwise complete medium would permit growth of *L. leichmannii* 313: thymidine, adenine desoxyriboside, hypoxanthine desoxyriboside, cytosine desoxyriboside, desoxyribonucleic acid, ascorbic acid, or small amounts of refined liver extracts, used as a source of vitamin B_{12}. Fewer substances were effective for some of the other organisms tested, and one, a strain of *Lactobacillus delbrueckii*, specifically required thymidine or its mother substance, desoxyribonucleic acid (32).

It is clear from these results that under the conditions so far investigated the requirement of lactic acid bacteria for vitamin B_{12} is far from specific, and this fact undoubtedly explains many of the difficulties encountered in attempts to assay for this substance in natural materials with these organisms. The nutritional equivalence of various desoxyribosides and vitamin B_{12} may indicate

that the vitamin functions in these organisms to permit synthesis of the desoxyribosides, which are required for formation of desoxyribonucleic acid. When these are supplied preformed, the catalyst is no longer essential (29, 30, 32). This hypothesis fails to account for the growth promoting action of ascorbic acid. It is possible that under the reducing conditions induced by this compound, those organisms which respond to it are able to synthesize vitamin B_{12}. Appropriate reducing agents other than ascorbic acid are also effective in eliminating the vitamin B_{12} requirement of these organisms (25), so that ascorbic acid per se should not be considered as a growth factor for them.

Euglena gracilis var. *bacillaris* cannot utilize thymidine in place of vitamin B_{12} (26). This organism may become useful, therefore, in the assay of this vitamin.

Pyridoxamine phosphate and pyridoxal phosphate.—During fractionation of an unidentified factor required for growth of a strain of *Lactobacillus helveticus* and one of *L. acidophilus*, McNutt & Snell (33) noted that the growth-promoting activity of concentrates was destroyed by exposure to light, and by incubation with a crude preparation of malt phosphatase. Trial of the phosphorylated forms of the light labile vitamins revealed that synthetic pyridoxal phosphate was highly active. Pyridoxamine phosphate, prepared from pyridoxal phosphate by transamination with glutamic acid (34), was six to ten times more active than was pyridoxal phosphate and gave a dose-response curve very similar in shape to that given by crude malt extracts. About 0.4 mμg. of pyridoxamine phosphate per ml. of medium sufficed to permit half-maximum growth. The preparation was approximately one million times more active than a crude extract of malt in promoting growth of the organism and appeared to account quantitatively for the growth promoting action of the crude material. The natural occurrence of pyridoxamine phosphate had been previously demonstrated by Rabinowitz & Snell (34).

Bellamy, Umbreit & Gunsalus (35) have shown that all of the various forms of vitamin B_6 (pyridoxine, pyridoxal, and pyridoxamine) which are utilized by a given organism are converted by it to pyridoxal phosphate, which is the only functional form of the vitamin known. Since the unphosphorylated compounds, pyridoxamine and pyridoxal, were completely ineffective in promoting growth (33) of these two organisms, it is evident that a

breakdown in the specific mechanism for phosphorylation of these substances has occurred. This recalls the requirement of certain strains of the gonococcus for thiamine monophosphate or thiamine-pyrophosphate (36). Attention should be called to the differences in availability of the phosphorylated forms of vitamin B_6 to various test organisms. For *Saccharomyces carlsbergensis*, neither pyridoxal phosphate nor pyridoxamine phosphate approach the growth promoting action of the unphosphorylated compounds; for *Streptococcus faecalis*, pyridoxal phosphate has very low activity, but pyridoxamine phosphate resembles pyridoxamine or pyridoxal in activity (34). These differences probably result from differences in permeability of the cells to the phosphorylated vitamins.

Guanylic acid and other nucleotides.—Cheldelin & Riggs (37) reported in 1946 that a certain strain of *Lactobacillus gayonii* failed to grow in a medium which supported excellent growth of many other lactic acid bacteria, e.g., *L. casei*. Addition of liver, yeast, or other crude materials permitted excellent growth. The factor was concentrated about 40-fold from liver, but not identified. Hutchings, Sloane & Boggiano (38) reported the unidentified factor to be stimulatory rather than essential for growth. It was destroyed at pH 4.0 by incubation with fungal enzymes such as clarase or takadiastase, suggesting the action of a phosphatase. This suggested the possible identity of the factor with nucleotides, and an ammonia-hydrolyzed preparation of yeast nucleic acid proved highly active. Each of the individual nucleotides of yeast nucleic acid—adenylic, guanylic, cytidylic and uridylic acids—proved active, about 15 to 30 μg. per ml. being required to permit half-maximum growth. The mixed nucleotides of the hydrolyzed nucleic acid were more active than any single nucleotide. In contrast to guanylic acid, guanosine was inactive, and the growth-promoting power of the other phosphorous compounds decreased on incubation with phosphatases in proportion to the inorganic phosphate liberated (38). Here again, a defect in a specific phosphorylation mechanism is indicated and results in a requirement for the intact nucleotides for growth. The equivalence of four different nucleotides in promoting growth when added individually remains unexplained. The observation is similar to that discussed earlier, where anyone of several desoxyribosides permitted growth of *L. leichmannii*.

Putrescine, spermidine, and spermine.—Lwoff & Lwoff showed

in 1937 that the V-factor required by *Hemophilus parainfluenzae* for growth on peptone media was identical with coenzyme I or II (39). Bass *et al.* (40) subsequently found that some members of this genus required growth factors in addition to those known in 1941. A strain of *H. parainfluenzae* studied by Herbst & Snell (41) did not grow in a synthetic medium containing known amino acids, vitamins, and growth factors, but grew if small amounts of yeast extract, orange juice, or other natural materials were added. Fractionation of yeast extract and orange juice showed the active material to be a strongly basic compound, and putrescine (1,4-diaminobutane, tetramethylene diamine) was eventually isolated and shown to be the active growth factor in orange juice. Spermidine and spermine, both of which contain the 1,4-diaminobutane residue, were almost as active on a molar basis as putrescine in promoting growth. Since these compounds, like putrescine, are widely distributed in natural materials (42) they undoubtedly contribute to the growth-promoting activity of such materials for *H. parainfluenzae* under the assay conditions. The compounds are quite active, less than 1.0 μg. per 10 ml. of medium being sufficient to permit half-maximum growth. Ornithine, cadaverine, trimethylenediamine, and a variety of other substances showing a structural relationship to putrescine were without growth promoting activity (41).

Putrescine is known to be produced by many bacterial cultures through the action of ornithine decarboxylase. A requirement for putrescine would imply that this enzyme was lacking in this particular organism. The essential nature of putrescine for growth of this organism and the wide distribution of this and related compounds in nature point to a metabolic role of fundamental significance for the compound. The finding emphasizes that the function of ornithine decarboxylase (and perhaps of other decarboxylases) in life processes is considerably more fundamental than merely to aid in maintenance of an internal pH favorable for growth, or to provide a convenient source of carbon dioxide for the organism (43).

It is interesting to note that Silverman & Evans (44, 45) as early as 1943 postulated that spermine and spermidine might play an essential metabolic role in bacteria. They were led to this suggestion by the observation that inhibition of growth of *E. coli*

by atabrine and quinine could be alleviated by either of these compounds. Snell (46) reported that these compounds also prevented toxicity of propamidine for lactic acid bacteria over a limited range of concentrations. Both groups found the effect nonspecific, however, since synthetic polyamines such as triethylenetetramine or tetraethylenepentamine were also effective in the reversal. Miller & Peters (47) confirmed the antagonistic action of spermine and spermidine to atabrine toxicity in *E. coli*, but were unable to obtain complete reversals; putrescine was ineffective. Because of the low order of antagonistic action, they discounted the possibility that the two amines were essential metabolites for the cell. The finding that these compounds are essential metabolites for *H. parainfluenzae* (41), however, indicates that the original interpretation of Silverman & Evans relative to the mode of action of atabrine and similar compounds deserves reconsideration.

Coenzyme A and its degradation products.—During a study of the pantothenic acid requirement of *Acetobacter suboxydans*, Sarett & Cheldelin (48) noted that some extracts of natural materials showed greater growth promoting activity than would be expected on the basis of their pantothenic acid content, as determined with *L. arabinosus*. The substance responsible for the enhanced activity could be extracted from fresh liver or heart muscle; indirect evidence based upon the release of β-alanine from concentrates of the factor by acid hydrolysis and its growth promoting action for *A. suboxydans* in a pantothenic acid-free medium indicated that it was a conjugated form of pantothenic acid (49, 50). Concentrates possessed about twice the activity for *A. suboxydans* which would be indicated by their pantothenic acid content; growth with the conjugate was also considerably more rapid than with free pantothenic acid. The concentrates were ineffective in promoting growth of *L. arabinosus* in a pantothenic acid-free medium, and pantothenic acid was not liberated from them by digestion with takadiastase and papain.

These properties coincide with those reported for coenzyme A, a combined form of pantothenic acid essential for enzymatic acetylations in living tissue, and which is reported by Novelli, Kaplan & Lipmann (51) to account quantitatively for the bound pantothenic acid of animal tissues and many bacteria. Cheldelin

and co-workers (50), however, found their concentrates (in contrast to coenzyme A) to be inactive in catalyzing acetylation of sulfanilamide by tissues. The discrepancy was clarified by Novelli, Flynn & Lipmann (52), who found that purified coenzyme A had the same growth promoting properties for both *A. suboxydans* and *L. arabinosus* as did the concentrates of Cheldelin and co-workers (50). Treatment with an enzyme of liver extract destroyed the coacetylase activity, but did not liberate pantothenic acid and did not decrease the growth promoting properties for *A. suboxydans*. Subsequent treatment of this product with intestinal phosphatase liberated pantothenic acid in a form available to *L. arabinosus* and destroyed the "extra" growth promoting action for *A. suboxydans*. Coenzyme A and the partial degradation product produced from it by an unidentified enzyme of liver should thus be considered as stimulatory growth factors for *A. suboxydans*. The chemical structure of the coenzyme is not yet known; it contains pantothenic acid, esterified phosphate, and additional unidentified groups.

Although coenzyme A is essentially inactive as a source of pantothenic acid for *L. arabinosus*, all of the pantothenic acid taken up by this organism appears in the cell as coenzyme A (51). This is similar to the findings that thiamine pyrophosphate and pyridoxal phosphate, though essentially inactive in promoting growth of yeast in thiamine- or vitamin B_6-deficient media, are formed from these vitamins by the cells and are required preformed as essential growth factors by other organisms. This is usually taken to indicate pronounced differences in permeability of various cell membranes to the phosphorylated vitamins, although other explanations are also possible.

Unidentified Growth Factors

A large number of ill-defined stimulatory effects of crude materials on growth of various microorganisms in purified media has been described; in a few cases the growth factors have been fairly well defined from the standpoint of their effects on growth, and considerable effort to purify and identify the active materials made. Unidentified growth factors in both categories have been listed together with the test organisms in reviews by Peterson & Peterson (8), Koser (53), and Snell (54). Where such reports appear in the older literature, it is probable that reinvestigation of the test organism in its relation to the vitamins and growth factors

now known might result in identification of the growth factor with known compounds. Only those unidentified factors with well-defined growth effects, and which have been the subject of recent investigations, will be discussed below.

Strepogenin.—A summary of early work on this factor, which is essential or stimulatory to growth of several streptococci and lactobacilli, and evidence indicating a relationship of the active substance to glutamine and asparagine, have been given elsewhere (54). The most clear-cut evidence for such a relationship is cited by Wright & Skeggs (55), who first found that enzymatic digests of casein were a potent source of the factor. These authors found that asparagine replaced such digests for a strain of *Streptococcus lactis*, but neither asparagine, glutamine, or a mixture of the two would do so for a strain of *S. faecalis*. The enzymatic digest, however, was much more active for *S. lactis* than asparagine. It was suggested (55) "that asparagine or glutamine or both may suffice as growth factors for certain streptococci but that they are involved in the structure or synthesis of more highly active compounds which function in the nutrition of the more fastidious strains." The latter compounds were thought to be those supplied by the enzymatic digests of casein. Sprince & Woolley (56) cited evidence indicating the identity of these stimulatory factors with those effective for hemolytic streptococci and *L. casei*, named the factor "strepogenin," and recommended a short-time assay with *L. casei* for its determination.

Pollack & Lindner (57) and Chu & Williams (58) pointed out that glutamine showed growth stimulating activity for *L. casei* under conditions which closely resembled those recommended for strepogenin assay, and Woolley found by direct assay that glutamine had strepogenin activity 50 to 200 times that of an arbitrary liver standard (59). Glutathione also has activity 4 to 10 times that of this standard (59). In contrast to that of glutamine and glutathione, however, the growth promoting activity of enzymatic casein digests is not destroyed by autoclaving (57, 59, 60), and all of the evidence indicates that the growth factor is a heat-stable peptide, which can be liberated from a variety of proteins by enzymatic hydrolysis (60). Sprince & Woolley (60) found digests of insulin to be the most potent natural source, with an activity some forty times that of the crude liver standard, i.e., approaching that of equal weights of glutamine and surpassing that of glutathione.

Following treatment with nitrous acid or dinitrofluorobenzene, insulin no longer gives rise to digests with growth promoting activity, indicating that some of the free amino groups of insulin [which are due solely to glycine, phenylalanine, and lysine (61)] are present in an amino acid which is a component of the strepogenin molecule (62). Since some concentrates of strepogenin contain no phenylalanine or lysine, glycine appears to be this amino acid. Woolley also observed that lycomarasmin, a tomato-wilting agent produced by *Fusarium lycopersici* which appears to be a substituted glycyl-asparagine (63, 64), antagonized the growth promoting action of enzymatic hydrolysates of casein for *L. casei*. It was postulated that this substance might be a naturally-occurring, structural analogue of strepogenin and owed its inhibitory properties to its interference with the utilization of strepogenin (65).

On the basis of these and similar findings, Woolley (59, 65) synthesized a number of peptides containing glutamic acid and tested them for strepogenin activity. Six of these were found to possess activity ranging from 1 to 0.1 times that of an equal weight of the standard liver extract. In order of decreasing activity these were: serylglycylglutamic acid, glutamyltyrosylglutamic acid, glycylserylglutamic acid, alanylglycylglutamic acid, glycylalanylglutamic acid, and glycylglutamic acid. Like the activity of protein digests, and in contrast to that of glutathione and glutamine, the activity of these peptides was not altered by heating with the medium (59). The low activity of L-serylglycyl-L-glutamic acid for *L. casei* was confirmed by Krehl & Fruton; a number of other peptides containing glutamic acid were inactive (66). The most active of the peptides, however, is less active on a weight basis than a crude tryptic digest of casein, and far less active than such digests of insulin. It is clear, therefore, that strepogenin as it occurs naturally does not correspond in structure to any of these compounds. In view of the high activity of glutamine, it would appear reasonable that the naturally occurring substance might be a glutamine-containing peptide, rather than one containing free glutamic acid.

Daniel and co-workers (67, 68) report that *L. casei* requires enzymatic casein digests (or glutamine) *plus* factors present in concentrated liver extracts (presumably the animal protein factor or vitamin B_{12}) and in whey for maximum growth over short time

intervals. The stimulatory action of the latter factors for this organism has not been observed by other workers; the difference very probably lies in the relatively small inoculum and the short sterilization time employed by Daniel *et al.* In a second paper from the same laboratory, Peeler *et al.* (69) cite evidence which they believe shows that glutamine is responsible for the entire "strepogenin" activity of enzymatic casein digests for *L. casei*. The basal medium used, however, contained no glutamic acid, and since this amino acid (or glutamine) is essential for growth of *L. casei*, the conclusion would appear invalid.

The Leuconostoc citrovorum factor.—Sauberlich & Baumann (70), in attempting to find an organism suitable for the microbiological determination of alanine, found that *L. citrovorum* failed to grow on a synthetic medium satisfactory for *L. mesenteroides*. The nutritional defect was remedied by addition to the medium of crude liver extracts, or of very small quantities of refined liver extracts, which then supported rapid and heavy growth. The responsible factor did not appear to be vitamin B_{12}, since preparations having an effect equivalent to this vitamin in animal nutrition were relatively ineffective for the microorganism, whereas yeast extract, which is very low in vitamin B_{12}, was as active in promoting growth as some refined liver extracts. Furthermore, direct trial of crystalline vitamin B_{12} has shown it to be inactive,[2] and Lyman & Prescott have separated the two completely by electrolysis in 0.02 *M* acetic acid (125). The factor was not identical with that required by *Lactobacillus bulgaricus* (see below). An interesting feature of the work was that a slow and incomplete growth response occurred when relatively large amounts of pteroylglutamic acid (folic acid) were added to the medium. Thymidine, previously shown to permit growth of this organism in a semisynthetic medium (31), was somewhat more effective than folic acid in promoting growth, but the rate and extent of growth with thymidine were also much less than that with refined liver extracts. It appears from these results that this substance is metabolically related in some unknown manner to folic acid, thymidine, and possibly to vitamin B_{12}. Nothing is known of the chemical nature of the growth factor.

The Lactobacillus bulgaricus factor (LBF).—Williams, Hoff-

[2] Personal communication from Dr. C. A. Baumann.

Jørgensen & Snell (71, 72) describe the conditions for assay, certain properties, and a procedure for the partial purification of an unidentified factor essential for growth of a strain of *L. bulgaricus*. The richest source of the factor found was yeast extract, although the substance is widely distributed in natural materials. By extraction with butanol, followed by adsorption and elution from charcoal and magnesium silicate, concentrates were obtained which were 150 to 300 times as active as yeast extract in promoting growth of the test organism. About 0.4 μg. of such concentrates per ml. of medium sufficed to permit maximum growth. At this stage of purity, it is somewhat more effective than the most active of the known amino acids in promoting growth, but somewhat less active than the least active of the vitamins. It could not be identified with any of a large variety of compounds of physiological importance and appeared to differ from other growth factors so far described. Ergostanyl acetate, which has been reported to cure an antistiffness syndrome in guinea pigs (73), partially replaced the factor, but growth with this compound was slow and never reached maximal levels. This substance was only about one-three hundredth as active as the best concentrates from yeast, and hence could not be the active factor in the concentrates. The growth factor was stable at room temperature to variations in pH between 2 and 10; at autoclave temperatures it is gradually destroyed near neutrality, and rapidly destroyed at the extremes of pH. It has neither strongly basic nor strongly acidic properties Several other strains of *L. bulgaricus* and one of *L. helveticus* required the growth factor, and preliminary evidence indicated it was widely required by the more fastidious organisms of the lactic group (72).

Protogen.—In their successful analysis of the nutritive requirements of *Tetrahymena geleii*, Kidder & Dewey have shown that this ciliated protozoan can be grown in a medium containing known amino acids and vitamins (many of which are required by the organism) provided a supplement consisting of the filtrate from lead acetate precipitation of many crude materials, and termed Factor II, was added to the medium (74, 75). Stokstad *et al.* (76), by application of fractional precipitation with silver, succeeded in separating Factor II into two components, termed Factors IIA and IIB, respectively. Kidder & Dewey (77), by adding purified preparations of Factor IIA to the medium, have shown that Factor IIB can be replaced by a mixture of high levels of pyridoxine (or much

lower levels of pyridoxal or pyridoxamine) and copper and iron salts. The only uncharacterized growth essential in the medium for these organisms is, then, Factor IIA, which has been named "protogen" because of its essential role for protozoa. Stokstad *et al.* (76) have further fractionated protogen and found it to exist in two forms with equivalent growth-promoting properties, but with different chemical properties. The distribution of this substance in several natural materials was determined. A variety of physiologically active materials, including strepogenin and vitamin B_{12}, failed to replace it in the nutrition of *Tetrahymena* and available evidence indicates that it is a new and distinct growth factor. In contrast to the *L. bulgaricus* factor, protogen is stable to hydrolysis at 120°C. with 2 N NaOH or 1 N HCl. The only procedure which destroyed the activity was hydrolysis with 8.0 N H_2SO_4 at 120°C.

Stimulatory factors for Streptococcus faecalis.—Several papers have appeared (78, 79, 80) describing the occurrence in natural materials of substances which markedly stimulate the rate of growth of *S. faecalis* R when this organism is grown from small inocula in media containing the known growth factors required by it. Since the substance is not essential for growth, the effect decreases and eventually disappears as the period of incubation is increased. Essentially nothing is known of the chemical nature of the responsible factor(s) which is variously reported as heat labile (78, 79) or heat stable (79, 80). Evidence that the organism is responding to more than a single substance is provided by the variable stability of the factor to heat, and the failure of the assay to yield quantitative results for the potency of one natural supplement in terms of another (77).

It should be pointed out that under similar conditions, i.e., with much smaller inocula than normally used, and restricted periods of incubation, the growth rate of almost all of the lactic acid bacteria can be materially increased by the addition of crude natural materials to synthetic media which contain all of the growth essentials (25).

Inhibitory Compounds and Apparent Nutritive Requirements

It is well known that the inhibitory action of many (though by no means all) compounds on growth of bacteria results from their

interference with the utilization or formation of materials which are essential for growth of the cell. Where such a mechanism exists, the inhibitory effects of the drug can frequently be alleviated by the addition of an excess of the compound with the utilization or formation of which the drug interferes. Under these conditions, the "antagonistic" metabolite appears as an essential nutrient for the cell, whereas normally it may be synthesized by the cell. Several reviews of metabolite-antimetabolite relationships provide adequate illustrations of these observations (81, 82), and Shive and co-workers (83, 84, 85) have developed the concept into a useful method for suggesting the course of biochemical transformations within the cell.

The extent to which such antagonistic relationships contribute to the nutritional requirements of organisms as determined under ordinary laboratory conditions is not always appreciated. A striking example is provided by the work of Washburn & Niven (86) with *Streptococcus bovis*. This organism grew well in a medium containing appropriate salts, sugar, vitamins and L-arginine. Single additions to this medium of isoleucine, leucine, threonine, norleucine, or alanine completely prevented growth. Further single additions of valine, glutamic acid, cystine, or methionine in appropriate amounts fully restored growth to the original levels. The amino acid "requirements" of this organism for growth quite obviously depend upon the basal medium in which the requirement is determined; depending upon the conditions the organism appears to require none, or any one of several, amino acids. A mixture of phenylalanine and tyrosine, but neither one singly, was inhibitory to this organism; this inhibition was overcome by tryptophane. Thus an apparent requirement for a growth factor in a given medium does not necessarily mean that the organism lacks the ability to synthesize that substance. Additional examples of this type are the observations (*a*) that the amount of serine required for growth of *L. casei* and *L. delbrueckii* depends upon the amount of threonine in the medium (87); (*b*) that the amount of glutamic acid required by *L. arabinosus* for growth depends upon the aspartic acid content of the medium (88); (*c*) that under certain conditions phenylalanine is required for growth of *E. coli* if tryosine is present in the medium, although the organism grows well if neither amino acid is present (89); (*d*) that some yeasts grow well

in the absence of thiamine, but addition of this vitamin inhibits growth and this inhibition of growth is alleviated by addition of vitamin B_6 (90). In each of these cases, an imbalanced nutrient solution has resulted in a qualitative or quantitative change in the requirement for additional nutrients. Similarly, the toxic effect of citrate for various lactic acid bacteria reported by Campbell & Gunsalus (91) was found by MacLeod & Snell (92) to result from a deficiency of manganous and magnesium ions induced in many of these organisms by the complex-forming action of citrate. Although a direct requirement for magnesium ion by *L. casei* could not be shown, this ion was essential for growth in the presence of citrate (92). Application of these principles should provide an alternative method to the use of naturally-occurring or artificially-produced mutants for the detection of new products of importance to metabolism. The discovery that *p*-aminobenzoic acid was an essential metabolite for bacteria was made in this manner, i.e., through the observation that natural materials counteracted the toxic action of a drug which acted in a manner not understood at the time (93). More recently, Shive *et al.* (28) isolated thymidine from liver extracts by virtue of the fact that it counteracted toxic effects of methylfolic acid. Only later was it shown to be directly required by many organisms (29 to 32). Silverman & Evans (44, 45) suggested that spermine or spermidine might be important metabolites because these materials counteracted the toxic action of atabrine; the suggestion finds its experimental verification in the observation that these substances or putrescine are growth essentials for *H. parainfluenzae* (41).

A variety of species of bacteria which are normally sensitive to streptomycin become resistant to this antibiotic when cultured in its presence. A smaller number comes to require this compound for growth (94 to 98). The observation recalls the development of a sulfonamide-requiring mutant of *Neurospora* (99). In the latter case, it was found (100) that the organism overproduced *p*-aminobenzoic acid, which in the absence of sulfonamides was indirectly toxic and prevented growth. Sulfonamides in sufficient amount prevented this toxicity, and hence permitted growth. A similar explanation for the development of streptomycin-requiring mutants would appear more logical than to assume development of a requirement for streptomycin per se, as postulated by Paine &

Finland (98). The observation that a compound is essential for growth under a given set of conditions is thus not indisputable proof that it normally functions in metabolism.

Interrelationships of Essential Nutrients

As the nutritional requirements of microorganisms become better known, it becomes increasingly clear that the magnitude of the requirement for any nutrient is not a constant, but may vary greatly depending upon the kind and amount of other ingredients in the medium. Antagonistic effects, such as those discussed above, contribute to such variation. Frequently, however, the variation is a direct reflection of the metabolic use served by a given material. For example, it has been known for several years that folic acid could be replaced as an essential nutrient for *L. casei* or *S. faecalis* by a mixture of purine bases and thymine (54). The latter substances did not permit synthesis of detectable amounts of folic acid by the cells (101), but in the presence of folic acid cells do synthesize purine bases and thymine [i.e., desoxyribonucleic acid (102)]. The desoxyribonucleic acid content of such cells is as high or higher when thymine and purine bases are supplied as when folic acid is supplied (102). All of the evidence thus indicates that the only essential role of added folic acid (in the media on which these studies were made) was to catalyze synthesis of thymine and purine bases, and if these are supplied preformed, then folic acid is no longer a nutritional essential (101, 102). Inhibition studies lead to the same conclusions (103).

Similarly, biotin is no longer a nutritional essential for lactic acid bacteria if aspartic acid and oleic acid are added to appropriate media, and these facts have been interpreted to mean that biotin normally functions in the synthesis of these two compounds, which are essential structural components of the cell. When these are supplied preformed, biotin need no longer be added to the medium (53, 104, 105).

A third illustration is provided by vitamin B_6. Lyman and co-workers (106) showed that *L. arabinosus*, which in complete media grows without vitamin B_6, requires this vitamin for growth in the absence of any one of the following amino acids: threonine, lysine, alanine, arginine, phenylalanine, tyrosine, serine, histidine, or aspartic acid. The mechanism by which a mixture of these

amino acids renders vitamin B_6 nonessential for this organism is not known; it is probable, however, that the vitamin functions as a catalyst in their synthesis, and when any one of them is omitted from the medium and hence must be synthesized, the requirement for vitamin B_6 is increased to the point where it must be added to the medium to permit growth. A similar explanation has been shown to explain the nutritional equivalence of D-alanine and vitamin B_6 for *L. casei* and *S. faecalis*. Holden, Furman & Snell (107) found that when cells of these organisms were grown with D-alanine in place of vitamin B_6 they contained only traces of the vitamin, i.e., D-alanine was not serving as a precursor to permit synthesis of the vitamin. When the cells were grown with vitamin B_6 and no D-alanine, however, they contained D-alanine demonstrating that this D-amino acid is necessary for growth of these bacteria and that one of the normal functions of vitamin B_6 is to catalyze its synthesis (108).

All of these interrelationships are very pronounced, representing the difference between no growth and excellent growth. More detailed study will undoubtedly reveal a variety of less marked, but equally significant effects of one nutrient upon the requirement for another. Several such relationships between vitamin B_{12}, desoxyribonucleosides, and ascorbic acid were noted earlier in this review.

Carbohydrate Requirements

Several recent papers emphasize the preference of various organisms for specific carbohydrates as energy sources for growth. Camien, Dunn & Salle (109) examined 22 carbohydrates or carbohydrate derivatives as energy sources for 23 different lactic acid bacteria. For the great majority, glucose was as suitable as any other carbohydrate, in accordance with common belief. A strain of *Lactobacillus buchneri*, however, failed to utilize glucose, but grew very heavily on either xylose or arabinose, and less heavily with fructose. No other organism failed to utilize glucose, but several produced much higher titratable acid with xylose or arabinose as the carbohydrate.

Snell, Kitay & Hoff-Jørgensen (110) observed a strain of *L. bulgaricus* which failed to grow readily in glucose-containing media, but which grew rapidly with lactose. Equimolar mixtures of glu-

cose and galactose were no more effective than glucose alone and did not approach lactose in growth promoting ability. After a long lag period, however, the organism fermented both monosaccharides, and subcultures from these cultures fermented glucose readily. In addition to lactose, many other β-galactosides (but no α-galactosides) were effective in promoting rapid growth, i.e., the specificity is not for lactose per se, but for the β-galactosidic linkage. A stimulatory effect of autoclaved monosaccharides on the rate of fermentation of β-galactosides could be duplicated by addition of pyruvic acid. When β-methyl-D-galactopyranoside was the energy source, pyruvic acid was essential for rapid growth (110).

The problem of isolating pure cultures of thermophilic cellulose-fermenting bacteria has hitherto not been solved satisfactorily. McBee (111) has now shown, however, that pure cultures can be readily obtained if enrichment cultures are plated from a cellulose-containing medium to one containing cellobiose as the sugar. Previous attempts have failed because glucose was the carbohydrate used. The pure cultures fail to ferment glucose, but ferment cellobiose readily. Glucose is not inhibitory to growth, since cellobiose can be utilized from cellobiose-glucose mixtures. β-Glucosides other than cellobiose were not tested as an energy source.

The ready utilization of certain disaccharides (glycosides) for growth, as contrasted to the unavailability of their component monosaccharides, poses an interesting problem relative to the mechanism of utilziation which has not been solved. The examples emphasize anew the limitations imposed on the type of bacteria isolated by the nutritive substrate employed [cf. also (112)].

MISCELLANEOUS

Streptococcus bovis requires only biotin among the vitamins, and no amino acids, for growth in a synthetic medium. It is stimulated by thiamine, pantothenic acid, nicotinic acid, glutamic acid, and arginine, which are necessary for subculture of most strains (113). It is thus unique among streptococci studied thus far in the simplicity of its requirements. A detailed study of the nutrition of *Lactobacillus pentosus* (114) showed it to resemble other lactic acid bacteria, particularly *L. arabinosus*, in growth requirements.

No chemically defined medium gave growth as rapid as a natural medium. The vitamin requirements of 23 lactic acid bacteria, as reported by Shankman *et al.* (115), should be considered as minimal requirements only. Kitay & Snell have shown (116) that several cultures employed by the former workers required additional vitamins for growth when smaller inocula were used or when cultures grown from heavy inocula were subcultured. A review of the nutritional requirements of lactic acid bacteria appeared (117).

Heavy inocula of *Bacillus larvae* grow in a defined medium, but growth does not continue on subculture (118), indicating a requirement for substances in addition to the known vitamins, amino acids, and purine bases of the medium. Thiamine and a purine base are essential; several amino acids were stimulatory and may prove essential when it becomes possible to initiate growth from smaller inocula. *Microbacterium lactis* has been cultured in a synthetic medium (119). Gerhardt & Wilson (126) describe the interrelationships of essential nutrients for *Brucella abortus* in a synthetic medium. Although ammonium salts serve as a sole nitrogen source, DL-asparagine supports better growth; a mixture of glycerol and lactate provides an excellent energy source.

The nutritional requirements of the Reiter treponeme, a nonpathogenic organism somewhat resembling the pathogenic *Treponema pallidum*, have been reinvestigated (120, 121). The organism grows in a medium containing acid-hydrolyzed casein (or a mixture of amino acids), several vitamins, a reducing agent, and serum albumin (120). Arginine, acetic acid, any one of a number of sulfhydryl compounds, and crystalline serum albumin were identified as essential for growth (121); yeast extract, an enzymatic casein digest, and other components of the medium supply the unidentified growth essentials. Acetic acid could be replaced by a number of related compounds, including acetaldehyde, pyruvic acid, and ethanol. Survival of virulent cultures of *T. pallidum* in a complex nutrient medium was favored by 5 per cent of carbon dioxide; no multiplication, however, has yet been obtained *in vitro* (122). The essential role played by traces of carbon dioxide in the nutrition of a variety of organisms has been somewhat clarified by the observations that it can be partially replaced as a nutritive essential for *E. coli* by many of the dicarboxylic acids of the isocitric acid

cycle, and their amino acid precursors, aspartic and glutamic acids (123, 124). Lwoff & Monod (123) indicate that unidentified factors in yeast extract are very effective in replacing carbon dioxide for this organism. A heightened carbon dioxide tension is essential for growth of *L. arabinosus* in vitamin B_6-free media lacking certain amino acids (106). The effectiveness of dicarboxylic acids in eliminating this requirement has not been determined.

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CONSTITUENTS OF VIRUSES

By C. A. Knight

The Virus Laboratory, University of California, Berkeley, California

Interest in the nature of viruses is great not only because of their potential capacity to wreak havoc on almost all forms of living matter, but also owing to the apparent similarity of some viruses and genes, and the possible bearing that viruses may have on the fundamental nature of life itself. Certainly, viruses appear at present to be the simplest substances possessing attributes of life. Outstanding in this respect are some of the plant viruses, which have been demonstrated by Stanley and associates (1, 2) and by Bawden & Pirie (3, 4) to be solely nucleoprotein in composition. The chemical analysis of such nucleoproteins assumes special significance, for unlike the case with more complex agents, such as bacteria, rickettsiae, or even many animal viruses, all of the biological activity of a simple plant virus can presumably be attributed to a single chemical compound, that is, to a specific nucleoprotein. However, the analyses of all viruses are important in establishing the nature of these disease agents. Moreover, it is to be expected that a thorough knowledge of the composition of viruses will aid, perhaps critically, in the solution of problems of host-virus relationship.

While the complete chemical analysis of a virus is clearly desirable in principle, in practice it can be most difficult to achieve. Accomplishment of this aim is dependent, first, upon the ability to obtain highly purified preparations of virus, and next, upon the availability of methods and tools for analysis.

Purity of virus preparations as a basis for analysis.—Since viruses are characterized by their ability to reproduce only within living cells, one must macerate the cells to effect release of the virus, unless, as in the case of many bacteriophages, infection causes disruption of the cells. In either instance, the problem is to separate the virus from cellular debris and to concentrate and purify it until it is essentially free of nonviral material. The feasibility of obtaining sufficient quantities of virus purified adequately for chemical analysis is predicated largely upon the availability of suitable amounts and kind of starting material, a reasonable

stability of the virus, and in most cases, the ability to measure virus activity with reasonable accuracy. Then, on the assumption that one, or a combination, of the methods currently employed for fractionating biological materials (1) will yield a concentrate rich in viral activity, one is faced with the task of demonstrating that this concentrate consists mainly of virus.

To establish conclusively the identity of a given substance with the virus is, as Stanley has noted, next to impossible (5); on the other hand, the failure to detect impurities by a number of tests, together with evidence of homogeneity by several criteria, can in many cases provide reasonable assurance that the material under examination is essentially pure and consists of virus. Some of the tests relied upon in this respect include constancy of properties in successive preparations and in preparations from different sources, homogeneity in sedimentation behavior, electrochemical homogeneity, serological purity, and other tests summarized by Lauffer & Stanley (6). None of these tests alone can establish the purity of a virus, but the results of a number of different tests considered together can frequently furnish an adequate basis for judging the quality of a given preparation. It should be pointed out that problems in establishing the purity of viruses are by no means unique to this class of substances, but apply also to other biologically active, complex entities, such as enzymes and certain hormones, whose identity cannot yet, owing to chemical complexity, be confirmed by synthesis. Progress in all these fields involving so-called macromolecules was undoubtedly accelerated by the timely development of methods and tests for purity.

At least some of the plant viruses can be considered foreign agents in their respective hosts in the sense that no significant quantities of nucleoprotein resembling these viruses are obtained from healthy plants [Bawden (4)]. This point is both of practical and of theoretical interest. In the purification of such viruses, plant pigments appear to comprise the main source of impurity, and these, by virtue of color, act automatically as indicators of their removal, which is accomplished in some cases readily, and in others with difficulty. Instances have been observed recently by Takahashi (7) and by Stanley (1) in which macromolecular constituents similar to the viral particles in sedimentation characteristics have complicated purification of certain Brassica viruses and the potato yellow dwarf virus. Also, a most interesting

circumstance has been reported by Markham & Smith (8), who observed that purified preparations of the nucleoprotein of turnip yellow mosaic virus contain approximately 20 per cent of material which appears to differ from the virus mainly in its lack of nucleic acid and of infectious capacity.

It is notably among the animal viruses that the greatest difficulty with nonviral particles has been encountered. For example, tissue particles which fall into the 10 to 300 mμ class, comprising the size range for viruses in general, have been isolated by Taylor and co-workers (9) and by Cohen (10) from chick embryos, and by Knight (11, 12) from chick allantoic fluid, and from mouse lungs. In addition, nonviral components have been detected by Kabat & Furth (13) in preparations of the agent of fowl sarcoma and leukosis, by Curnen, Pickels & Horsfall (14) in certain preparations of pneumonia virus of mice, by Duffy & Stanley (15) working with Japanese B encephalitis, by Glaser & Stanley (16) with the virus of silkworm jaundice, and also by Cohen (17) in preparations of bacteriophages. In some of these cases, it appears simply impossible to obtain highly purified preparations of virus by currently available methods. In other cases, the use of special techniques, such as the adsorption of influenza virus on, and its subsequent elution from, erythrocytes, has proved of decisive importance [Knight (12)].

A finding which may prove to be of significance in the understanding of the nature of animal viruses is the discovery by Cohen (10) and by Knight (11, 12, 18) that preparations of influenza virus, which are reasonably homogeneous serologically, electrochemically, and in sedimentation behavior, possess, apparently as an integral component, an antigenic moiety characteristic of the host. Attempts to remove this component from the viral particles by a variety of means have thus far been unsuccessful. Hence the inclusion of host antigen in the viral particles by some presently obscure mode of attachment seems well established and constitutes a marked difference between influenza virus particles and those of tobacco mosaic virus, for example. The most highly purified preparations of the latter give no indication by serological means of the presence of host antigens, even when tested by the extremely sensitive anaphylactic test (19).

On the basis of the available experimental evidence, Knight (18) was unable to conclude whether the host antigen present in

influenza virus particles has some essential function, perhaps pertinent to the host-virus relationship, or whether this antigen becomes incorporated simply as an impurity characteristic of the environment in which the virus was produced. On the other hand, Rivers (20) took the viewpoint that the host antigen is present in such preparations as an impurity. However, his choice of the case of pneumonia virus of mice (PVM), described by Curnen and associates (14 21, 22), to support this opinion seems inappropriate, since in contrast to influenza virus, the preparations of PVM have been relatively crude and none of them has yet been shown to be chemically, physically, or serologically homogeneous, or to be incapable of further fractionation into viral and nonviral components. Hence, if homogeneous preparations of PVM in the so-called "free virus" state can be obtained, the possibility still remains that they will be found to contain antigens characteristic of normal mouse lung.

From the foregoing discussion it should not be inferred that all animal viruses are expected to contain components related to the host. However, despite the temptation to do so, neither the identity of a virus nor the homogeneity of a viral preparation can be inferred, but must be established by rigorous testing; and in a number of instances it would seem especially that straight-forward serological techniques have been neglected in testing viral preparations for the presence of normal tissue constituents.

Another factor which can serve to complicate the purification of viruses from a chemical point of view is the possibility that low molecular weight substances may dissolve in or be adsorbed on the viral particles. An example of these is cholesterol, which has been found in the lipid fraction of most animal viruses [Beard (23)]. Hoagland, Smadel & Rivers (24) have shown that cholesterol can be removed from elementary bodies of vaccinia by extraction in the cold with ether without affecting virus activity and conclude, therefore, that this substance is not an essential constituent of the virus. It is possible that closer investigation of various viruses will reveal other substances in this category. The occasional adsorption of pigment by plant viruses has already been mentioned, and this possibility also arises in the cases of some animal viruses, such as Shope papilloma virus, which is ordinarily extracted from warts rich in melanin pigment [Beard (25)]. However, neither Beard and associates (26) nor the author have experienced serious

difficulty in separating virus from pigment in this instance.

In summary, it appears that viral preparations can contain ostensibly nonviral material in one or more of three different ways. The preparations may be contaminated with varying amounts of independent, nonviral particles, i.e., host tissue constituents; the viral particles may contain as integral components antigens characteristic of the host; and the viral particles may be adsorbed on or have adsorbed on them a variety of nonviral substances. Insofar as chemical analysis of viruses is concerned, the presence of 5 per cent or less (the practical limits for detection of impurities by many currently employed methods) of nonviral substances may at present be of little importance. In the case of the incorporation of host materials into viral particles, the quantitative tolerance is much larger so long as a constant amount is uniformly incorporated, as it apparently is with influenzal viruses; indeed, such combinations may eventually be shown to represent the simplest form in which the virus exists. Much useful information of a general sort can be obtained by analysis of the latter viral preparations, although it is obvious that the fine points relating chemical constitution to viral activity are best investigated by chemical studies on the simplest highly purified viruses, such as tobacco mosaic virus.

Methods for analysis of viruses.—It has been mentioned that the timely development of methods for testing homogeneity aided immeasurably in the advancement of chemical virology. However, once the general chemical nature of viruses was established, further progress in this field was dependent upon the availability of practical methods for analysis of the major constituent parts of viruses, namely, protein, nucleic acid, lipid, and carbohydrate. Again, fortunately, recent developments in some of these fields of analysis have provided imperfect but useful tools for continuation of the chemical attack on viruses. For example, as recently as six or eight years ago it would have been difficult if not impossible to account for 95 per cent or more of a protein in terms of its constituent amino acids. Hence, the apparently complete analysis of β-lactoglobulin in 1945 by Brand and co-workers (27) established a landmark in protein chemistry, which was followed by similar analyses of other proteins, including those of some strains of tobacco mosaic virus [Knight (28)].

There is now available a variety of methods for amino acid

determination which include colorimetric and spectrophotometric procedures, microbiological assays, isotope dilution, and various forms of chromatography. The advantages and limitations of these and other methods have been discussed in a series of papers presented before the Section of Physics and Chemistry of the New York Academy of Sciences (29, 30).

Very recently the techniques of chromatography and spectrophotometry have been combined by Hotchkiss (31) and by Vischer & Chargaff (32, 33) to provide a promising method for the qualitative and quantitative determination of the constituent purine and pyrimidine bases of nucleic acids. Other approaches to the analysis of nucleic acid components have been made by Merrifield & Dunn (34), and by Loring and associates (35) employing microbiological assays, and by Tinker & Brown (36) using the Craig counter-current distribution technique. Separation of nucleotides, nucleosides, and related substances by ion-exchange methods [Elmore (37), Harris & Thomas (38), and Cohn, (39)] and by partition chromatography on starch [Reichard (40)] have also been reported. With these various techniques, nucleic acid chemistry should advance with great strides.

The quantity and general nature of the fatty substances comprising the lipid fraction of a virus can be estimated indirectly by means of the method of Kirk, Page & Van Slyke (41).

No entirely satisfactory general method appears to be available for the quantitative determination of carbohydrate in viruses. However, an approximate value can be obtained by employing the orcinol-sulfuric acid procedure of Tillmans & Philippi (42). The qualitative, and in some cases quantitative, identification of carbohydrates can be accomplished with minute amounts of the partially purified carbohydrate fraction of a virus by means of a spectrophotometric technique described by Gurin & Hood (43), refined by Seibert & Atno (44), and applied to the analysis of a virus by Knight (45).

Chemical composition of viruses.—Following the discovery of the nucleoprotein nature of tobacco mosaic virus, it was natural to look for protein and nucleic acid in preparations of other viruses. These constituents have been found thus far in all highly purified preparations of viruses, and, in addition, lipid, carbohydrate, and a few other substances have been detected in the cases of some viruses [Knight (46)]. In general, there can be said to be a

gradation in chemical complexity from the plant viruses, which appear to consist solely of nucleoprotein, to vaccinia virus, which Hoagland and co-workers (47) found to consist of protein, desoxypentosenucleic acid, lipid, copper, biotin, and flavin. Furthermore, there appears at present to be a rough correlation between size and chemical complexity of a virus. As a consequence, the viruses at one end of the scale resemble bacteria in morphology and chemical constitution whereas those toward the other extreme are very much like familiar protein molecules. All of this seems to support the concept of Stanley (48) that viruses fit smoothly into a scheme of things which progresses with no sudden transitions from atoms to mammals.

The amount of lipid found in viral preparations varies from about 1.5 per cent for the Shope papilloma virus [Taylor *et al.* (49)] to approximately 50 per cent for the virus of equine encephalomyelitis [Taylor *et al.* (50)]. The T_2 bacteriophage was reported by Taylor (51) to contain about 2 per cent of lipid in the form of neutral fat, but since the preparations of phage analyzed were not tested for host antigens, the possibility exists that this small amount of fat was present in nonviral particles such as those described by Cohen (17). The same argument can be applied to the lipid of the papilloma virus preparations. The lipid fractions of vaccinia [Hoagland (47)] and influenzal viruses [Taylor (52)] have been found to contain neutral fat, phospholipid, and cholesterol.

Both pentose and desoxypentosenucleic acids have been found in viruses [Knight (46)]. While individual viruses have been found to contain a characteristic amount of nucleic acid, a tremendous range in quantity has been noted among different viruses. For example, the virus of Newcastle disease has been reported by Cunha and colleagues (53) to contain a quantity of nonlipid phosphorus equivalent to only about 1 per cent nucleic acid. On the other hand, Stanley (54), Cohen & Anderson (55), and Taylor (51) have found approximately 40 per cent nucleic acid in tobacco ringspot virus and in T_2 bacteriophage. In between these extremes lie equine encephalomyelitis, influenza, tobacco mosaic, and vaccinia viruses, possessing in the neighborhood of 4 to 6 per cent nucleic acid, Shope papilloma virus with about 9 per cent, and tobacco necrosis, tomato bushy stunt, alfalfa mosaic, southern bean mosaic, and silkworm jaundice viruses possessing 15 to 21

per cent [Knight (46), Bergold (56)]. It is to be hoped that future investigations will reveal the significance of this great spread in nucleic acid content of different viruses.

All plant viruses tested thus far have been reported to contain only pentosenucleic acid, but both types have been found in animal viruses. The nucleic acids of the Shope papilloma [Taylor *et al.* (49)] and vaccinia [Hoagland (47)] viruses appear to be exclusively the desoxypentose type, whereas that of equine encephalomyelitis virus [Taylor *et al.* (50)] seems to be solely pentosenucleic acid. In the cases of influenza, T_2 coli bacterial, and Newcastle disease viruses it has been claimed that both pentose and desoxypentose-nucleic acids are present.

Beard (23) listed a value of 1.5 per cent desoxypentosenucleic acid for the PR8 strain of influenza virus, citing the work of Taylor (52), who, however, reported 2.1 per cent. Beard (23) and Taylor, working in Beard's laboratory (51), both conclude that influenza virus contains only desoxypentosenucleic acid. However, Taylor's data appear to contradict this conclusion, for they include the statement that, in addition to a "weakly positive" test for desoxy-pentose (57), a positive test for pentose was obtained with Bial's reagent (52). Furthermore, the difference between Taylor's total phosphorus and lipid phosphorus values (52) shows clearly that there must be around 5 rather than 2 per cent nucleic acid in his preparations, unless there are phosphorus-containing constituents present in addition to nucleic acid and phospholipid. No such phosphorus-containing moiety in such preparations has been reported by any of the workers in this field. On the other hand, Knight (11, 12) obtained color reactions indicative of the presence in purified preparations of influenza virus of both types of nucleic acid, and later obtained from highly purified viral preparations concentrates which were shown to be rich in nucleic acid by their chemical composition and their spectrophotometric behavior (45). These concentrates were found to give unequivocally positive tests for desoxypentose by two different reactions and to give, in addition, a strongly positive test for pentose in the Bial reaction. Furthermore, tests made with crystalline ribonuclease indicated the presence of pentosenucleic acid. Totalling the amounts of pentose and desoxypentosenucleic acids calculated to be present in preparations of PR8 influenza virus, a value of about 5 per cent was obtained, which is a figure in reasonable accord with the non-

lipid phosphorus of these preparations. Therefore, it would seem that strong evidence exists for the presence of both types of nucleic acid in highly purified preparations of the PR8 strain of influenza virus, and most likely in other types of influenza virus as well.

Cohen & Anderson (55) reported that purified preparations of T_2 bacteriophage contained 37 per cent of desoxypentosenucleic acid, and later Cohen (58) stated that regardless of the medium from which the virus originated, at least 99 per cent of the total phosphorus of his preparations could be accounted for by the desoxypentosenucleic acid content of the preparations. Opposed to these findings are those of Taylor (51), who reported the presence of both types of nucleic acid in his preparations of T_2 bacteriophage and who claimed, moreover, that the contents of these nucleic acids varied with the medium in which the viral host cells had been grown. He obtained values of 40.3 and 44.6 per cent of desoxypentosenucleic acid and 6.6 and 1.3 per cent of pentosenucleic acid depending upon the medium. On one hand, it can be argued that it is difficult to detect small amounts of pentosenucleic acid in the presence of large amounts of the other type. On the other hand, it is known that the phosphorus distribution method upon which Taylor largely based his conclusions is capable of irregularities, and the control run by Taylor (51) did not correspond with the phage analysis in proportions of constituents. Moreover, Cohen (17) has recently found that some of his preparations of variants of T_2 coliphage may contain small amounts of pentosenucleic, as well as desoxypentosenucleic acid, but that the pentosenucleic acid is associated with contaminating host particles which can be separated from the phage by serological means. Therefore, it is questionable that T_2 bacteriophage contains both types of nucleic acid. Some doubt is also cast on the significance of the quantitative differences in desoxypentosenucleic acid content of Taylor's two types of preparations, and on the discrepancy between his and Cohen's values, owing to the finding just mentioned and the further discovery by Cohen (59) that as much as 30 per cent of the nucleic acid of some preparations appears to be adsorbed on the exterior of the phage particles and can be separated enzymatically from them without diminution of virus activity.

The third virus which possibly contains both types of nucleic acid is Newcastle disease virus. Aside from the results of qualitative tests indicating the point just mentioned, little information on the

nucleic acid of this virus was presented by Cunha and associates (53), and the purity of the viral preparations has been questioned by Beard (23) in whose laboratory the work was done. However, it appears that in addition to its curious morphology, Newcastle disease virus is singular in its low content of nucleic acid. It is possible, of course, that this virus has not yet been obtained in its simplest form, and that eventually a fraction richer in nucleic acid, yet possessing essentially all of the viral activity, will be isolated.

Loring (2) demonstrated the general similarity in chemical composition of tobacco mosaic virus nucleic acid and yeast nucleic acid; more recently, Schwerdt & Loring (60) concluded from the results of several physical and chemical tests that each of three nucleotides isolated from virus nucleic acid is identical with its analogue from yeast ribonucleic acid. The preliminary experiments of Markham & Smith (61) employing paper chromatography have indicated that the nucleic acid of turnip yellow mosaic virus contains the same kinds of purines and pyrimidines as the ribonucleic acids of yeast and of tobacco mosaic virus. Positive tests for the purines and pyrimidines expected in desoxypentosenucleic acid were obtained by Hoagland and associates (62) with nucleic acid isolated from vaccinia virus. The results of color reactions made by Cohen & Anderson (55) on T_2 bacteriophage indicated that the nucleic acid of the virus contains equivalent amounts of purines and pyrimidines.

As mentioned previously, methods are just becoming available for the quantitative determination of the constituents of nucleic acids. However, even with such methods, the task will be complicated by the necessity of separating the nucleic acid from the virus in an essentially intact condition. In contrast to nucleoproteins of the sperm type, viral nucleic acids are frequently firmly bound to protein or to other viral constituents and may require vigorous treatment, such as subjection to strong alkali, to cleave them from the virus. Fortunately, in the case of tobacco mosaic virus, it was found possible by Cohen & Stanley (63) to separate the nucleic acid from the viral nucleoprotein by the relatively mild procedure of heating very briefly in the presence of salt. The nucleic acid preparations thus obtained were viscous, spontaneously birefringent, and contained particles with an estimated molecular weight of approximately 300,000. More recently Markham & Smith (8)

have described an even milder method for obtaining pentose-nucleic acid from turnip yellow mosaic virus. It seems likely that nucleic acid preparations such as the latter two will prove valuable not only for the information they can be expected to yield regarding the chemical nature of the viruses from which they were derived, but also for establishing fundamental facts concerning the chemistry of nucleic acids in general. In the latter respect, the nucleic acid obtained from highly purified preparations of virus probably conforms more closely to the desired standard of a single molecular species than do nucleic acid preparations obtained by currently employed methods from such heterogeneous sources as yeast or thymus cells.

It should be mentioned that the failure to detect nucleic acid in a viral preparation by spectrophotometric methods does not prove its absence, for the absorption of ultraviolet light by viral particles can, as in the case of influenza virus [Knight (45)], completely mask the specific absorption due to nucleic acid, particularly if the latter is present in small amount. It is possible, of course, that eventually a virus will be found which contains no nucleic acid. However, no case of this sort has yet been encountered; on the contrary, the assumption that a virus will contain nucleic acid is, for example, apparently aiding in attempts by Atlas and co-workers (64) to isolate and characterize viruses of the common cold, although it is not at all certain that these workers are measuring nucleic acid in their tests. The finding that polyhedral bodies of silkworm jaundice were very low in phosphorus and hence in nucleic acid content, encouraged the belief that the virus constitutes only a small part of these bodies, and led to the fractionation of them and isolation by Bergold (56) of small rod-shaped units rich in nucleic acid, which, as judged by several criteria, appear to be the virus.

The potential importance of nucleic acid in virus reproduction seems especially well exemplified in the results obtained by Markham & Smith (8) with the turnip yellow mosaic virus. Purified preparations of this virus were found to consist of approximately 80 per cent nucleoprotein and 20 per cent of free protein, and the two materials appeared to have the same electrophoretic mobility, isoelectric point, crystalline form, and serological properties. However, the data indicated that only the nucleoprotein possesses virus activity.

Another point worthy of note is that nucleases apparently do not always attack nucleic acids combined with protein, such as viral nucleoproteins. Thus, it was shown by Loring (65) that ribonuclease has no enzymatic effect on tobacco mosaic virus, and by Cohen (59) that desoxyribonuclease has no effect on the integrally combined nucleic acid of T_2 bacteriophage. Possibly, it may be argued in the latter instance that the enzyme was kept from its substrate by a viral membrane. Nucleic acids freed from viruses are, as expected, readily attacked by the appropriate nuclease. These findings suggest that specific enzymes need available the same chemical groups that are in some cases involved in the combination of nucleic acid with protein.

Viruses have been found to vary in protein content from a low of about 50 per cent, characteristic of equine encephalomyelitis virus [Taylor *et al.* (50)] to 94 per cent, found in the viruses of the tobacco mosaic group [Knight (46]. The few virus proteins examined thus far for amino acid content have been found to be composed of 14 to 18 of the amino acids commonly observed in proteins. In general, the composition of viral proteins has contrasted with that of the classical partners of nucleic acid, the protamines and histones of Kossel (66). The latter proteins are characterized by their basic nature, attributable to a high content of basic amino acids, such as arginine and histidine. On the other hand, the proteins of strains of tobacco mosaic, influenza, and Shope papilloma viruses have been found by Knight (28, 67, 68) to possess a preponderance of neutral and acidic amino acids. These viruses possess far less nucleic acid than the sperm nucleoproteins. However, a bacterial virus and tobacco ringspot virus, both known to contain large quantities of nucleic acid, have also been found by Polson & Wyckoff (69) and by Knight (68) to contain only ordinary quantities of the basic amino acids. Also, dicarboxylic rather than basic amino acids have been found by Markham and associates (70) to predominate in turnip yellow mosaic virus, which contains about 28 per cent nucleic acid. Hence, it appears that the proteins of viruses are not protamines or histones but are acidic proteins which probably resemble in a general manner those present in certain bacterial nucleoproteins [Sevag & Smolens (71)].

The development of methods for protein analysis has made it possible for Knight (28) to account for substantially all of the pro-

tein of some strains of tobacco mosaic and of other viruses in terms of their constituent amino acids. Most of the values in these analyses were obtained by means of microbiological assays, with which a complete analysis can be made on a total of 60 mg. or less of virus. The accuracy of such assays, as Stein (72) has noted, is based more on empirical than on theoretical grounds. Moreover, these assays share the uncertainties characteristic of most protein analyses, namely, the incomplete knowledge of what occurs during hydrolysis of a protein, and the partial dependence, at least, upon commercial amino acids of unequal purity as standards. Therefore, it seems probable that a number of the values secured by means of microbiological assay will stand correction as better methods and standards become available; on the other hand it should be recognized that results obtained by microbiological methods have been demonstrated in many cases to agree very well with the values obtained by other procedures, including methods based on solubility product or isotope dilution techniques [Snell (73)]. It is also clear that for the purposes of comparing compositions of similar materials, such as strains of a virus, the values secured by methods such as microbiological assays are as useful as demonstrably absolute values.

Knight, working in Stanley's laboratory, has been attempting to compare the chemical compositions of strains of viruses with the hope of elucidating by inference the chemical nature of virus reproduction and virus mutation. In one of these investigations (28) 40 differences in protein composition between tobacco mosiac virus and seven of its strains were discovered. Most of these differences in protein composition were in the relative proportions of certain amino acids, but it was also found that one of the strains (HR) contains two amino acids, histidine and methionine, not present at all in TMV or in the other strains examined. Therefore, it appears that mutation of tobacco mosaic virus can be accompanied by changes in the amino acid content of the virus. Similar differences were also found between two types of influenza virus [Knight (67)]. The results of microbiological assays indicated that these strains of influenza virus contain approximately the same amounts of 12 different amino acids, but that they differ significantly in content of arginine, glutamic acid, lysine, tryptophane, and tyrosine.

T_2 bacteriophage was found by Cohen & Anderson (55) to

contain 1.45, 2.77, and 2.86 per cent, respectively of tryptophane, tyrosine, and arginine. Polson & Wyckoff (69) have reported values for 14 amino acids found in the closely related coliphage, T_4. Their results, obtained by a method based on paper chromatography, are not expressed in terms directly comparable to those of Cohen & Anderson, but the arginine value, at least, looks as though it differs significantly from that recorded for T_2 coliphage.

Analyses made by Knight (45) indicate that the particles representing PR8 and Lee influenza viruses contain a polysaccharide constituent composed of mannose, galactose, and glucosamine units. The estimated content of this component was 4 to 5 per cent of the virus, but it should be noted that the total quantity of carbohydrate in current influenzal virus preparations has not been settled. The values reported by Taylor (52) and Knight (12) for the PR8 strain are 12.5 and about 6 per cent, respectively. Co-operative investigations of this discrepancy have thus far failed to resolve it.

Studies made on rickettsiae by Cohen & Chargaff (74), by Tovarnickij and associates (75), and by Cohen (76) indicate that these agents are at least as complex chemically as some of the animal viruses, for they have been found to contain protein, nucleic acid, lipid, and carbohydrate combined in complex forms.

To summarize, it can be said that, at present, viruses in general seem to be composed of various combinations and amounts of nucleic acid, protein, and frequently lipid, with polysaccharide, vitamins and other materials appearing as yet only in special cases. Nucleic acid and protein can be singled out from this list of constituents, for these two substances, combined to form specific nucleoproteins, appear to comprise the minimum chemical requirement for virus activity, and moreover, nucleic acid and protein constitute the only components common to all viruses.

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ACTIONS OF ANTIBIOTICS IN VIVO[1]

By Windsor C. Cutting

Department of Pharmacology and Therapeutics, Stanford University School of Medicine, San Francisco, California

PENICILLIN

Penicillin continues to be the most unique and satisfactory therapeutic weapon known. Its edge is sharp but it can scarcely harm the user. Recent studies of its effects in living organisms can perhaps be most easily discussed when divided into those affecting microorganisms and those affecting the host organism.

Action on Microorganisms

Antibiotic action.—As with most drugs, the fundamental mode of action of penicillin is not clear. Two superficial facts are well known, i.e., that gram positive bacteria are more susceptible than gram negative and that the division and growth of bacteria are impeded with the formation of bizarre forms when early death or lysis is not produced. Pandalai & George (1) associate these effects with an interference with the nucleic acid metabolism of the bacteria, which coincides with the observation that gram positiveness is associated with the presence of magnesium ribonucleate. Gale and co-workers in a series of papers (2) show that gram positive organisms (staphylococci) require an external source of glutamic acid, and that the assimilatory mechanism is blocked by penicillin. The same microbes, made highly resistant to penicillin, become like gram negative bacteria and are able to synthesize their own glutamic acid. These two views may be compatible if it is assumed that the glutamic acid becomes a constituent of nucleoprotein.

A paradoxical effect has been reported by Eagle (3) and Pratt & Dufrenoy (4), in which high concentrations of penicillin, *in vitro*, are less effective against several common microorganisms than lower concentrations. Still lower concentrations, of course, are again less effective. The latter authors also note an enhancement of effect when traces of cobalt are present, perhaps because this element stimulates growth of the bacteria and thereby increases their susceptibility to penicillin.

[1] This review covers the period from January, 1947 to December, 1948.

No particularly new fields of action for penicillin have been found. Gram positive bacteria and the meningococcus and gonococcus, a few fungi, most spirochetes, and the viruses of the lymphogranuloma-psittacosis group of large viruses represent the most susceptible species.

Molecular species.—At least eight types, or species, of penicillin have been recognized (5), and chemical and *in vitro* differences extensively studied. For clinical use, only penicillin G is readily available, and when the various species are compared, either in animals or man, it contests well with penicillin X and is superior to penicillin K [Rothman-Kavka and others (6)]. The latter type suffers, not from lack of antibacterial potency, but from the manner in which it is handled by the body, probably through a greater degree of immediately inactivating adsorption on plasma proteins, as has been recently discussed by Thompsett *et al.* (7). The question of the enhancement of the activity of penicillin by impurities, including phenylacetic acid, has not been clearly answered, although it has been extensively discussed by Hobby *et al.* (8).

Resistance.—Some bacteria, particularly gonococci, were noted to become increasingly resistant to the sulfonamides as several years of clinical use went by. The fear that penicillin will share this fate is often expressed. The question is well discussed by Miller (9), who defined a resistant organism as one which "requires more antibiotic to prevent its multiplication than most other strains of the same bacterial species." There is, of course, a tremendous variation between different species. Miller believes that resistance will not greatly complicate clinical use, because it is seldom of high degree, and penicillin is commonly administered in excess of the minimum inhibitory concentration.

Nevertheless, certain bacteria, notably staphylococci, often become resistant *in vitro* on being grown in increasing concentrations of penicillin, or may become resistant *in vivo* during penicillin therapy. Other strains are naturally resistant. Spink & Ferris (10) describe increases in resistance, acquired *in vivo*, of as much as a thousandfold. This is associated with an increase in penicillinase production. Resistance produced *in vitro* tends to be less, to be associated with a loss of virulence, and not to be due to an increase of penicillinase production. The production of penicillinase does not appear to be entirely responsible for the resistance of gram negative bacteria, as stressed by Bondi & Dietz (11).

Gezon (12) in a series of papers has extended studies on resistance to include the hemolytic streptococci. Although there are group differences, the common human pathogens of group A tended to develop resistance (up to seventeenfold) when grown in increasing concentration of penicillin. The resistance usually was temporary and associated with a decrease in virulence.

This important problem, then, appears to be partially answered and the following working hypothesis may be tenable. Except possibly for staphylococci, it is anticipated that resistance will not be a serious clinical problem. Resistance may result from the growing out of a few naturally resistant organisms in a culture until they form the mass of the organisms, or may be induced in otherwise susceptible organisms through mutations which give rise to strains better adapted to resist penicillin. Finally, resistance produced *in vivo* appears to be more serious than that produced by a laboratory trick and more definitely related to an increase in the production of penicillinase.

Administration.—A presently debated problem concerns the administration of penicillin and how maximum effect may be obtained. For a number of years, great emphasis has been put on the search for new methods by which penicillin could be induced to remain in the blood for long periods. This action was based on the assumption that a high continuous plateau would produce the greatest antibiotic effect. This thesis has recently been challenged by several workers.

Jawetz (13) early demonstrated that there was a continuing antibacterial action of penicillin in the body, after it could no longer be demonstrated in the blood, and that an interval between injections of 12 hr. was not too long, in mice, to produce a satisfactory effect against hemolytic streptococcal infections. To be equally effective in a single dose, however the, quantity of penicillin had to be increased tremendously. His conclusion, therefore, was that repeated injections were necessary for economical use, but that the interval between injections could be quite long, and that it was not necessary that penicillin be constantly demonstrable in the blood. Presumably penicillin is still present in the tissues after it cannot be found in the blood, and even when it has disappeared entirely it may take some hours before bacteria are again actively multiplying in significant numbers. Gerber *et al.* (14) studied the penetration of penicillin into lesions and concluded

that high peaks of blood concentrations were more effective than a continued lower plateau.

Marshall (15) summarized the problem in a review and expressed the belief that efficient penicillin therapy in man could be obtained by means of 1 to 3, not immoderately large, daily injections of penicillin in an aqueous medium. This view agrees with the recent experimental work of Zubrod (16), Gibson (17), and White *et al.* (18), who studied various schedules of treatment in mice injected with hemolytic streptococci or pneumococci. At intervals up to 12 and even 24 hr., the total dose was the deciding factor, and not the frequency of injection. Eagle (19) stressed that the length of time during which penicillin-free levels could be permitted depended on the recuperative power of the organism. Thus, it would be shorter for pneumococci, which may recuperate in an hour than for spirochetes, which may remain depressed for 10 to 30 hr., but in any case, the anti-infective action appears to outlast the presence of the drug.

It would appear reasonable to expect that in humans an intramuscular injection of from 100,000 to 300,000 units, given twice daily, might suffice in the majority of infections with moderately susceptible microorganisms. With the smaller dose, a priming initial injection of 300,000 units might be in order. Whether the high peaks, so produced, would enhance the total effect, or only increase the waste of penicillin, is debatable. In infections with more resistant bacteria, as in some cases of bacterial endocarditis, larger doses would undoubtedly be necessary, though the interval might be lengthened over the usual 3 or 4 hr. It remains to be seen whether simple aqueous penicillin will replace the more complicated and somewhat more hazardous forms of depot penicillin.

Action on Host

As for many other new drugs, the natural history of penicillin in the body of the host has been scrutinized to a detail surpassing that for most older agents. Its entry, sojourn, incidental effects, and final departure have been investigated where ever accessible.

Administration.—Penicillin has been administered experimentally and clinically by almost every conceivable route, and its concentration then compared in different tissues and fluids. For such studies, only bioassay methods of analysis are available, which

increases the labor and decreases the exactness as compared to chemical methods available for many other drugs.

The usual route of administration is by intramuscular injection; less commonly by intravenous or subcutaneous paths. Other routes generally are aimed at obtaining desired concentrations in special tissues.

For intramuscular injection, both aqueous penicillin and repository or depot penicillin are used. As stated in an earlier section, there are active proponents of the use of aqueous penicillin in place of the more slowly absorbed types, but at present the latter are probably more commonly used. Many papers reporting the absorption characteristics of penicillin in oil and wax and procaine penicillin (20) in oil or in aqueous suspension are appearing. Of all these delaying types, the last one, namely procaine penicillin prepared with dispersing and stabilizing agents for aqueous solution, appears the most desirable because of the elimination of oil and wax, which may in some patients produce allergic or foreign body reactions. However, a certain incidence of reactions to the procaine must be expected.

Oral administration, most desirable from the standpoint of convenience, was early shown to be much less efficient than parenteral routes (21). This inefficiency is due in part to destruction by acid in the stomach, but this can be counteracted by enteric coating, or more consistently, by the addition of buffers such as sodium citrate. When thus protected, penicillin is carried in good quantity to the upper intestine and there is well absorbed. Blood levels are usually low, however, because excretion through the kidneys almost keeps pace with alimentary absorption. The net efficiency of oral administration, as compared to intramuscular injection, is seldom better than one-fifth that of the parenteral route. Nevertheless, for other than severe infections, the oral route is often a welcome and satisfactory alternative to injection therapy (22).

Another special means of administration is by inhalation of penicillin dust (23) or penicillin aerosol (24). This semilocal application is often used in the treatment of sinusitis, and in such chronic bronchial infections as bronchiectasis. Although favorable results are claimed, even exceeding those obtained by parenteral injection, the question is difficult of assessment because absorption from the aerosol mist may be considerable and so give the effect of an in-

jection. Sloan, Bain & Brucer (25) measured the depth of penetration of nebulized substances into the pulmonary tree of animals and showed the amount deposited on the walls decreased steadily from the pharynx down to the bronchioles and that none was demonstrable in the alveoli. They doubted that therapeutic concentrations could be achieved below the trachea. Laurent *et al.* (26) presented slightly conflicting observations. Penicillin was administered to rats by inhalation and by intramuscular injection, and the resulting concentrations in lungs and blood were compared. Penicillin concentration in the lungs was 60 times as high 1 hr. after inhalation as after injection, while the blood level at 1 hr. was the same by both routes. Therapeutic effects by the two routes in pneumococcus infection in rats were comparable (27). The short distance over which the penicillin must be carried to penetrate deeply into a rat lung makes comparison with the human difficult, and it is not justifiable to conclude from presently available evidence that there is a high alveolar concentration of aerosols in human patients.

Rectal absorption (28), vaginal absorption (29), and absorption by ion transfer (30) are hardly satisfactory except for local medication. Injection into arteries (31) and subcutaneous injection by "hypospray" (32) are still further variations on the route of administration. The latter is particularly interesting, and while not yet entirely practical, may prove to be a future means for the subcutaneous injection of many drugs. It consists of the forceful injection, in a fine jet, of small amounts of concentrated solution through the intact skin. The incidence of sensitization of patients is high after local application of penicillin, and, in fact, ointments and the like are usually inadvisable on this ground. The mechanism for this increased likelihood of sensitivity appears to lie in the ease with which antigenic combinations of drug, protein, and lipid can be produced in the skin. The "hypospray" would therefore be suspect until experience proved otherwise.

Distribution.—Penicillin was shown in early studies (33) to be widely distributed in the tissues and organs of animals, except for the nervous system. In view of continuing inhibition of bacterial growth in an infected host, after penicillin can no longer be measured in the blood, there has been renewed interest in the temporal relationship between penicillin in the blood and penicillin in the tissues. Schachter (34) clearly showed that penicillin was retained longer in the lymph, liver, and kidney of the dog than in

the blood. Usually it was present in the liver longer than in the lymph or kidney. This, then, supports the belief that a sustained blood level for clinical effect is not necessarily essential, and that injections may therefore be considerably spaced.

The poor penetration of the drug into the spinal fluid has led to the necessity in most infections of the meninges and central nervous system of employing intrathecal administration, or of changing to another drug, usually sulfadiazine. The latter is the usual recourse in meningococcic meningitis, for instance, but intrathecal penicillin is commonly used in pneumococcic meningitis, which tends to be a more serious and prolonged disease.

Boger and his co-workers (35) compared the cerebrospinal penicillin with the blood penicillin after intramuscular injection of the drug in patients with syphilis of the central nervous system. They found that no direct correlation could be made, but that, in general, perceptible amounts of penicillin appeared in the spinal fluid when the blood levels were in the high therapeutic range.

Excretion.—Because the kidneys excrete penicillin so rapidly, numerous attempts have been made to restrict this excretion and thereby increase and prolong blood levels. The attempts have included particularly the blocking of part of the renal tubular mechanism by diodrast, benzoic acid (36), *p*-aminohippuric acid (37, 38), and especially caronamide (4′-carboxyphenylmethanesulfonanilide) (39). Eagle & Newman (40), in a careful study, concluded that such methods were expensive and laborious, which is also the usual impression among clinicians.

Eagle & Newman (40) summarized present knowledge of the mechanism of urinary excretion of penicillin. They showed that in human subjects injected with single doses, or perfused continually with penicillin F, G, X or K, the renal clearance of the first three approximated the total renal plasma flow and was four or five times the glomerular filtration, as determined with inulin. Thus about 80 per cent of penicillin is excreted by the tubules. The renal clearance of K was one-fourth or one-half that of the other species, presumably due to binding or inactivation by plasma and tissues.

Toxic reactions.—Penicillin has almost no direct toxicity. An exception must be made for the guinea pig, which may succumb to what would be ordinary doses for other species, namely 250 to 1,000 units. Cormia *et al.* (41) report that this toxicity results from necrosis of the adrenal glands.

Untoward reactions of allergic nature are not uncommon, however. Fortunately they are seldom serious, and most reports are of only single cases. The skin is the organ usually involved. The commonest reactions follow local application of penicillin, but some follow parenteral administration.

Templeton, Lunsford & Allington (42) include the following as observed reactions: contact dermatitis after ointments, solutions, and lozenges; local reactions after intramuscular injections of repository penicillin; general reactions after systemic administration, including erythema, urticaria, serum sickness, phytids, and exfoliative dermatitis. The Arthus phenomenon may be produced in rabbits (43), but other reactions are uncommon. Fortunately, the dermal reactions in man often are ameliorated by the administration of antihistamines, although these agents do not influence the Arthus-type reaction in animals (44).

One other special type of toxicity is that produced by strong solutions of penicillin in contact with the central nervous system. The question was carefully studied in dogs by Pilcher, Meacham & Smith (45). Convulsions appeared regularly when a critical level of 300 units per cc. of spinal fluid was exceeded. This is far above the usual concentration produced by therapeutic intrathecal injections in man.

Although penicillin has been alleged to increase the coagulability of the blood, the observations of Weiner *et al.* (46) and of Dolkart *et al.* (47) fail to confirm this impression. In therapeutic concentrations there appeared to be no regular change in the coagulability of the blood.

The Herxheimer reaction requires separate consideration. Traditionally, this exacerbation of syphilitic lesions is presumed to result from the release of toxic substances from spirochetes by the action of therapeutic agents. With penicillin it is often striking and may make the secondary rash blossom brightly, with an accompanying but fleeting fever. The reaction in such patients appears to be without danger, and perhaps also in patients with neurosyphilis. In those with cardiovascular syphilis the threat is more serious, and fetal or placental reactions resulting in death have been reported. Initial treatment for a few days with penicillin in low dosage is advised in doubtful situations. The whole question was discussed in a recent symposium on venereal diseases (48).

STREPTOMYCIN

Unlike penicillin streptomycin is not an unalloyed delight to pharmacologist and clinician. It antagonizes certain bacteria, particularly of the gram negative group, toward which penicillin is relatively ineffective, but this is its only virtue. And even in this action, the net result of therapy is generally less striking than that of penicillin against microorganisms properly sensitive to it. In addition, streptomycin often produces resistance, and its toxicity on the host is by no means minimal. The situation has been improved by the introduction of dihydrostreptomycin, which produces less harm, but even this derivative still suffers badly in comparison with penicillin.

Action on Microorganisms

Antibiotic action.—More is known about which microorganisms are injured by streptomycin than about the essential action by which this effect is produced. Even the chemical structure has only recently been clarified.

The essential antibacterial action has been studied by Henry *et al.* (49). They postulated that streptomycin either inhibits an enzyme involved in carbohydrate metabolism, or inhibits its formation. The crucial action, however, does not yet appear to have been formulated. Hamre and co-workers (50) have shown that streptomycin has bactericidal action on young forms as well as old forms, thus contrasting with the action of penicillin which is limited to growing cells.

As with penicillin, the effectiveness of streptomycin *in vivo* is usually less than that *in vitro*. The typhoid bacillus, for instance, is susceptible in the test tube, but hardly touched *in vivo*. For most colon bacilli, a few micrograms of streptomycin per cc. of medium *in vitro* is lethal, but there are great strain variations. This variation is also marked in *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*, which may require from only one or two up to several hundred micrograms for killing. The tubercle bacillus is moderately susceptible, although variation in sensitivity, again, is considerable. There is relatively little effect against *Treponema pallidum* (51).

Particularly in tuberculosis, the use of adjuvants to enhance the action of streptomycin has been stressed. These have included

p-aminosalicylic acid (52), Promin (53), and potassium iodide (54), in each case reputedly with improvement in effectiveness.

Resistance.—Bacterial resistance is a major problem in the clinical use of streptomycin. Some microorganisms may become resistant to phenomenal degrees, almost overnight; other strains may be induced to become moderately resistant by being grown in increasing strengths of the drug. Whether these changes are due to mutations, or to overgrowth of a small percentage of naturally resistant variants, or both, is a current question. There has been one claim for the production of a "streptomycinase" by bacteria (106).

In 1946, Miller & Bohnhoff (55) showed that some strains of meningococci in four transfers increased in resistance to streptomycin from less than 1 unit to as much as 75,000 units per cc. These resistant strains retained their resistance *in vivo*. Similar effects were noted for gonococci, and by other authors (56, 57) for the tubercle bacillus, dysentery bacilli, and others. In 1947, Miller & Bohnhoff (58) reported the astonishing finding that some of the streptomycin resistant meningococci actually required the drug for growth.

The clearest demonstration of the presence of two types of resistance was made by Clark & Rantz (59). They showed that colon bacilli from the urinary tract might become resistant in small orderly steps when they were grown in low, but progressively higher, concentrations of streptomycin. Thus organisms which grew in 5 μg. but were killed by 10 μg. of the drug per cc. of medium could, in four steps, be made to grow in 20 μg. per cc. This was considered to be relatively unimportant clinically as therapeutic concentrations above this range could be constantly maintained. Other cultures, however, in the same length of time developed organisms resistant to 1,000 μg. of streptomycin. This is far outside the reach of therapy and makes the further use of streptomycin futile.

This strong tendency for the production of bacterial resistance makes brisk, intensive therapy desirable, to be abandoned if resistant forms appear. For many infections, most striking for tularemia, such a course is easy and appropriate. For tuberculosis, which resists easy and quick eradication, longer treatment appears to be desirable, and the early development of resistance is a tragedy. Fortunately, in many patients with tuberculosis the micro-

organisms do not become highly resistant in a three-month period of treatment. Thus, Sadusk & Swift (60) found that nine of 20 patients had resistant bacilli at this time while Fisher (61) found resistant forms in five out of 20 patients. At the start of treatment most of the tubercle bacilli were susceptible to 1 μg. per cc.; at the end, the most resistant were not killed by 2,000 μg. per cc.

Administration.—For systemic effect, the intramuscular route is usual, as with penicillin. Due possibly to somewhat slower excretion, the question of depot injections versus aqueous injections has not received as much attention as with penicillin, and there are, in fact, no preparations for slow absorption commercially available. The most effective interval for the injection of aqueous solutions has not been established but the general tendency is to lengthen the interval from the previously common 3 hr. to 6 or even 12 hr. Zubrod (62) has stressed the fact that the less frequent administrations are satisfactory, although Jawetz (63) showed that increased doses are needed with delayed and infrequent infections.

Dihydrostreptomycin.—This derivative of streptomycin is made by reduction with hydrogen. The antibacterial activity usually parallels that of streptomycin and resistance develops similarly, but the toxicity appears to be much decreased (64). It is customarily administered intramuscularly, in aqueous solution, in a dose of 0.5 to 1.5 gm. every 12 hr.

Action on Host

The actions of streptomycin on the host stand in striking contrast to those of penicillin in one principal particular—that of toxicity.

Administration.—Streptomycin is poorly absorbed when given by mouth, and it is therefore mainly given by injection, usually intramuscularly in aqueous solution. It is not inactivated in the gastrointestinal tract and is excreted in the feces virtually unchanged. It has been tried by this route against ulcerative colitis and as a means of preparation for surgical operations on the bowel, although apparently not with any striking qualitative differences from the effects produced by the poorly absorbed sulfonamides.

Like penicillin, streptomycin is occasionally given by other routes, intrathecally, by aerosol, and by local application. Although Bernard, Vreis & Grumbach (65) reported a relatively satisfactory penetration of streptomycin into the spinal fluid after

intramuscular injection, their practice agrees with that generally followed in this country, namely, supplementing intramuscular medication with intrathecal medication when needed, particularly in tuberculous meningitis. The current policy of the National Research Council (66) is to advise 25 to 100 mg. of streptomycin in a single, well diluted dose daily, when intrathecal medication is required.

Streptomycin, when given as an aerosol inhalation to rats (26), produces a high concentration in the lungs, just as penicillin does, but relatively much less is absorbed into the blood. Topical application of streptomycin, although effective, is usually inadvisable because of the danger of causing sensitivity in the host.

Distribution.—Marshall (67) developed a chemical method for the determination of streptomycin which allowed study of its distribution in the body. It appears to be distributed approximately according to the amount of body water. This agrees in general with the earlier work of Baggenstoss, Feldman & Hinshaw (68), who reported that after a series of large doses (0.8 gm. every 2 hr. for 9 doses) the following concentrations were found, in μg. per gm.: urine, 3,507; blood serum, 120; kidney, 173; lung, 42; spleen, 21; liver, 17; cerebrospinal fluid, 16; brain, 0.

Excretion.—Streptomycin appears in the bile, but the major route of excretion is by the kidneys. Marshall (67) showed that the renal plasma clearance in both man and dog is lower than the glomerular clearance, indicating that excretion is largely glomerular rather than tubular. For this reason, caronamide (39) cannot be used to decrease excretion, as it has been with penicillin. Nelson *et al.* (69) reported that a combination of streptomycin with trypan blue produced a complex which entered the liver and spleen better than streptomycin alone, and then was released slowly during many hours.

Toxicity.—The acute toxicity of streptomycin in animals is not excessive, although large, rapid intravenous doses produce muscular weakness and then coma. Chronic administration produces reversible fatty metamorphoses in the liver and kidneys of dogs and monkeys (70).

In man, several types of bothersome and disabling toxicity may develop. The most serious is a selective effect upon the eighth cranial nerve (71), uncommon with small doses given for only a few days, but affecting most patients given large doses over many

days, for instance, 2 gm. daily for 20 days. Tinnitus and vertigo are followed by a permanent diminution of labyrinthine function so that the ability to walk is lost until re-education allows substitution of visual control. Deafness appears less often, but is also permanent. The great hope for dihydrostreptomycin is that these dangers will be lessened, as appears to be the case. When a prolonged course of administration is in prospect, the patient should be tested before the drug is started for vestibular and auditory function, with repeated examinations throughout the treatment. Severe impairment of either function may be cause for discontinuance of the drug (72).

A second type of toxicity involves the kidney, producing proteinuria, hematuria, and sometimes uremia. Fortunately, this is not often extreme. Pain at the site of injection, sometimes requiring the inclusion of procaine hydrochloride in the injection, is another common manifestation.

The final type of toxicity is related to allergic sensitivity to streptomycin. Rashes, fever, and eosinophilia are occasionally observed after systemic administration. Dermatitis, both generalized and local, has followed contact with the drug (73, 74), and, as for penicillin, limits the local use of streptomycin.

TYROTHRICIN

Tyrothricin, the oldest of the antibiotics from the standpoint of current use, has been relegated almost entirely to local application. Systemic administration is impossible because of the hemolytic action of the agent. It is used considerably, however, in aqueous suspensions or creams, for pyogenic infections of the skin. It has a particular advantage here because sensitivity manifestations on the part of the patient appear to be much less frequent than with penicillin or streptomycin. The present day use of tyrothricin is summarized by Herrell (75).

BACITRACIN

Bacitracin was introduced by Johnson, Anker & Meleney in 1945 (76) as an antibiotic derived from the Tracy strain of *Bacillus subtilis*. It is a neutral substance, water soluble, relatively nontoxic, and heat stable, with greatest effect against gram positive bacteria. Further details of production and partial chemical characteristics have been recently described (77). It contains a number

of amino acids; the molecular weight is probably under 4,000. Several biological assay methods have been described (78, 79, 80).

The first extensive clinical report, by Meleney & Johnson, appeared in 1947 (81), recording the local use of the antibiotic in 100 surgical infections. The list of sensitive bacteria was much the same as for penicillin, but bacitracin appeared to be more effective than penicillin against some staphyloccoci and nonhemolytic streptococci. Bacitracin was not inhibited by blood or pus and no natural antagonist comparable to penicillinase was found. A second paper (82) reported successful use in a number of skin infections, with only rarely any development of slightly increased resistance of the microorganisms during treatment. It has also been used against ocular infections (83). Bacitracin was shown to have a strong antispirocheticidal effect (84) *in vitro*, but to be only one-tenth as effective as penicillin against experimental syphilis in rabbits. Systemic use in man is still little beyond the investigative state (85).

The pharmacological properties of bacitracin were studied by Scudi and co-workers (86, 87, 88). In mice and rats the drug was not highly toxic, although there was slight renal tubular necrosis in mice after large doses. Similar renal changes were found in monkeys but not in dogs. In the dog, bacitracin was not absorbed when given by mouth, nor recovered in the stool, and was therefore presumed to be destroyed in the alimentary tract. Following parenteral injection, the drug could still be detected in the blood after 8 hr. Little of the substance penetrated into red cells or into the spinal fluid. Attempts to produce anaphylaxis in guinea pigs failed. Excretion was studied by Eagle *et al.* (89), who found that the renal clearance of bacitracin approximated the glomerular filtration, both in rabbits and in man. Therapeutic levels in the blood persisted longer with bacitracin than with penicillin, again in both rabbits and man, presumably because of poorer total renal excretion.

Contact dermatitis may result from bacitracin (82), but the toxic potentiality which has limited its parenteral use in man has been renal damage. Albuminuria has been noted, but it has been suggested that the nephrotoxicity is produced by a contaminating substance, not bacitracin.

It is difficult to predict the future for bacitracin. The field of local antiseptics is crowded, and the outlook for systemic usefulness

depends upon elimination of its toxicity to the kidneys and further verification of its effect in cases where penicillin is of little value.

AEROSPORIN AND POLYMYXIN

In 1947, an antibiotic called aerosporin was obtained from *Bacillus aerosporus* in England, and another antibiotic called polymyxin from *Bacillus polymyxa* in the United States. These organisms are related, if not identical, and the antibiotics appear also to be closely related.

Aerosporin was described by Brownlee *et al.* (90, 91) as a basic peptide, with a chemotherapeutic activity *in vitro* toward a number of gram negative organisms 10 to 100 times that of streptomycin, but without activity against the tubercle bacillus. It protected mice from severe infection with *Hemophilus pertussis*, *Salmonella typhosa*, the colon bacillus, and others. Resistant strains were produced only with difficulty. When given by mouth in animals, it was not absorbed and eliminated sensitive bacteria from the alimentary canal. Upon parenteral injection, it disappeared promptly from the blood, necessitating an interval of 4 hr. between injections. It did not appear in the spinal fluid, bile, or urine after parenteral injection. Therapeutic doses were 0.2 to 0.4 mg. per kg. body weight. Aerosporin was more acutely toxic than streptomycin but the therapeutic margin was still adequate. Other types of toxicity, namely an antidiuretic action and damage to renal tubules, appeared to be due to impurities.

Polymyxin has been less completely described (92, 93) but follows the general description for aerosporin. In clinical use it appears safe and efficacious, though an adequate comparison with streptomycin and aureomycin has not yet been reported.

CHLOROMYCETIN

The antibiotic chloromycetin was described in 1947 by Ehrlich and his associates (94) as a crystalline substance isolated from cultures of a *Streptomyces*. It is bitter, relatively insoluble in water, but well absorbed from the alimentary tract. Smadel & Jackson (95) reported that chloromycetin had a beneficial effect on mice and embryonated eggs infected with a number of rickettsiae and viruses of the psittacosis-lymphogranuloma group. The drug was promptly tried by Smadel and co-workers (96) against epidemic typhus in Mexico, against scrub typhus in Malaya, and against

Rocky Mountain spotted fever (97) in the United States. Payne, Sharp & Knaudt reported its use against epidemic typhus in India (98). Although all these reports were on the basis of relatively few patients, they were uniformly encouraging. The clinical course was shortened and the severity of the disease greatly lessened. Typhoid fever has also been reported to be ameliorated by the drug (99). Administration is customarily by mouth, although the drug is given to some patients intravenously. Doses between 1 and 8 gm. daily have been used, so far without obvious toxic effects, except possibly for occasional nausea, which may have been related to the bitter taste of the drug.

From these preliminary reports it is impossible to predict the ultimate usefulness of chloromycetin, particularly as it will obviously compete with aureomycin, which has had a somewhat more extensive study, including more adequate periods of observation after cessation of treatment.

AUREOMYCIN

Aureomycin is an antibiotic of golden color, derived from the mold, *Streptomyces aureofaciens*. Preliminary studies indicate that it has an unusual range of action, including bacteria, rickettsiae, and the largest viruses. It is relatively stable, except in alkaline solutions; it is supplied as the hydrochloride which is easily soluble in water, producing an acid solution.

Action on Microorganisms

The antibacterial spectrum of aureomycin is broad. Against gram positive bacteria it is active, though usually inferior to penicillin; against gram negative bacteria streptomycin is possibly somewhat more active than aureomycin. Bryer *et al.* (100), for example, found that several strains of streptococci, pneumococci, and staphylococci were susceptible, *in vitro*, to about 1 μg. of the drug per ml., while the colon bacillus required about 5 μg. and some other gram negative organisms still more. Fifty times these concentrations were required in the presence of human serum, *in vitro*, although this is not necessarily the case *in vivo* due to other antibacterial forces in the intact host.

In a report made by a number of workers from several institutions at the New York Academy of Sciences, July 21, 1948 (101), aureomycin was shown to be effective against the viruses of the

psittacosis-lymphogranuloma group in experimental infections, and clinically against trachoma and several other viruses causing ocular infections, but to be ineffective against rabies, measles, influenza, poliomyelitis, and a number of other smaller viruses. It also appeared to be active against most rickettsial infections, including Q fever, and against spirochetes (105).

Finland, Collins & Paine (102) stated that aureomycin was bacteriostatic or bactericidal only against rapidly growing organisms, but no detailed clues as to mechanism of action have yet appeared. Attempts to demonstrate an aureomycin-inhibiting substance, similar to penicillinase, were unsuccessful. High degrees of anaerobiosis decreased its effectiveness. It was possible to induce moderate resistance, *in vitro*, but not to the same degree, nor as easily, as to streptomycin.

Aureomycin has usually been given by mouth, less often intramuscularly, and blood levels of less than 2 μg. per cc. have been effective clinically (103).

Action on Host

Aureomycin is well absorbed when given orally. The daily dose is up to 4 or 6 gm., divided into three or four doses (103, 104). In patients not able to tolerate oral medication, it has been given intramuscularly, but in smaller dosage, usually 20 mg. every 6 hr. or intravenously.

Distribution in the body has not been elaborately studied. It could not be detected (102) in bile from the common bile duct of man after oral administration, nor in the spinal fluid of dogs (100).

Collins *et al.* (103) studied the excretion of aureomycin. They found that urinary excretion was slow, with the highest concentrations (250 μg. per cc. after an oral dose of 0.75 gm.) appearing within the first 8 hr., but that less than 15 per cent of the total ingested was excreted in 2 days.

Aureomycin is not highly toxic. Intramuscular and intravenous injections produce some local irritation, but hourly conjunctive instillations of 0.5 per cent solution of aureomycin borate are well tolerated.

The toxicity was studied in animals by Bryer *et al.* (100). The LD_{50} on intravenous injection for mice is between 50 and 100 mg. per kg. body weight; on subcutaneous injection between 3 and 4 gm. per kg. Excessive doses in mice and dogs were followed by

anorexia, hyperpnea, tremors, paresis, and somnolence. Necropsy revealed no gross or microscopic abnormalities except local necrosis at the sites of injection. In man (102) the commonest complaints were looseness of the bowels and occasional nausea. Rash, fever, anemia, jaundice, or renal injury was not noted. When treatment with large doses was initiated in patients with brucellosis (104), an abrupt rise of temperature, sometimes with an accompanying drop of blood pressure and other evidence of shock, was frequently seen about 10 hr. after the first dose. This "Herxheimer" reaction appeared not to be of serious consequence, but led to initiation of therapy with a series of small doses of the drug.

A host of other antibiotics has been described, some dating back to Pasteur. A few, like pyocyamin, were used clinically for years before finally dropping from sight. Many other new or revived antibiotics than those mentioned in this paper show desirable *in vitro* activity. A few show promising *in vivo* activity, and remain for further exploration and possible exploitation.

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THE CELLULAR BASIS OF IMMUNITY

By William H. Taliaferro
Department of Bacteriology and Parasitology, University of Chicago, Chicago, Illinois

Once invading organisms or other antigenic materials have passed or have been introduced beyond the epithelial or mechanical barriers, their localization and disposal is a part of local (local inflammation) and general defense reactions. The main functional roles of the cells involved in these reactions are (*a*) to remove foreign material by extra- or intracellular digestion (phagocytosis) or to isolate it by a connective tissue wall (encapsulation); (*b*) to perform various reparative processes; and (*c*) to produce specific antibodies. With the exception of that part of repair which involves the parenchyma, the cells involved belong almost exclusively to the connective tissue, especially to the blood and lymph, to the reticular and loose connective tissues, and to the lamina propria and interstitial connective tissues of various organs including the brain. The cells are important to varying degrees in immunity because (*a*) they are ubiquitous in distribution or are easily mobilized via the blood stream; (*b*) they are phagocytic and/or possess potent digestive enzymes; and (*c*) they form a mesenchymal reserve in the postnatal vertebrate by retaining varying degrees of their embryonic power to develop into other cells of the connective tissue. These cells have other functions. As a whole, they take part in such diverse processes as intermediate metabolism, storage and mechanical support. A review of the functions of the reticulo-endothelial system is given by Jaffé (1) and of the leucocytes by Rebuck (2)

The present review stresses work of immediate interest to immunologists. It also outlines briefly the cells involved and their interrelationships because both subjects are fundamental to an understanding of the cellular aspects of immunity. The coverage, as a result, although largely limited to the last 10 years, has been selective, particularly as regards the latter aim, and includes some older work for purposes of orientation.

Connective Tissue Cells Involved in Immunity

The cells involved in immunity are listed in Table I together with the systems into which they have been incorporated (cf. 3)

and their mesenchymal potencies as used in this review (cf. 4, 5). Their origin and general behavior in inflammation and immunity are admirably summarized by Marchand (4) and Maximow (5, 6), who

TABLE I

CONNECTIVE TISSUE CELLS INVOLVED IN IMMUNE REACTIONS

I. Predominantly fixed cells			
A. Fibroblasts and endothelial cells			
B. Macrophages	R.E.S.,‡ restricted sense (Aschoff)		
1. *Reticular cells of reticular organs			
2. *Littoral cells of sinuses of reticular organs and of sinusoids of the liver, adrenal and hypophysis.			
3. *Adventitial cells (Maximow's undifferentiated pericytes)		Macrophage system (Metchnikoff)	
4. †Histiocytes of ordinary connective tissue and of the lamina propria and interstitial connective tissue of various organs, e.g., macrophages of skin, stroma cells of intestine, septal cells of lung, and glial phagocytes of brain.	R.E.S.,‡ broad sense (Aschoff)		Lymphoid-macrophage system (Taliaferro and Mulligan)
II. Free cells			
A. †Inflammatory macrophages			
B. †Intermediate polyblasts (cells transitional between nongranular leucocytes and inflammatory macrophages)			
C. Nongranular leucocytes			
1. †Monocytes (Ehrlich's transitional cells and large mononuclears)			
2. *Lymphocytes (including hemocytoblasts of myeloid tissue)			
3. Plasma cells			
D. Granular leucocytes			
1. Heterophils (granules characteristic of the species, e.g., neutrophils, pseudoeosinophils)		Microphages (Metchnikoff)	
2. Eosinophils			
3. Basophils			

* Marked mesenchymal potencies demonstrated.
† Restricted mesenchymal potencies demonstrated.
‡ Reticulo-endothelial system.

differed chiefly on the relative importance of the local tissue and blood as sources of inflammatory exudate cells. The role of cells in immunity has been reviewed by Perla & Marmorston (7). The role of the reticulo-endothelial system in immunity has been reviewed by Aschoff (8), Linton [(9) for protozoan infections], Jungeblut (10), and Jaffé (11).

Fibroblasts are widespread in connective tissue and endothelial cells line all nonsinusoidal vessels. Their functions in immunity largely involve the formation of connective tissue walls around massive accumulations of indigestible foreign material or repar-

ative proliferations such as the formation of new connective tissue. Fibroblasts in mammals behave as end cells but they may become reticular cells in regenerating spleens of birds, presumably with complete potencies for developing into other connective tissue and blood cells, according to Bloom & Taliaferro (12).

As used in this review, the term macrophage is a physiological designation for any large mononuclear cell which is not markedly basophil and can become phagocytic without essential change in morphology. Although predominantly fixed, many become free, especially in inflammation. As thus defined, macrophage is usually synonymous with histiocyte or reticulo-endothelial cell and the larger polyblasts. It is also frequently designated by other names according to the location and theory of hemopoiesis, e.g., adventitial cell or pericyte of the perivascular connective tissue; reticular cell of reticular organs; stroma cell of the lamina propria of such organs as the intestine; the stellate cell of von Kupffer in the liver, septal cell in the lung; and resting wandering cell, rhagiocrine cell and clasmatocyte in the skin and loose connective tissue in general. Reticular cells lining the sinuses of the reticular organs (spleen, lymph nodes, and bone marrow) and phagocytic cells lining the sinusoids of the liver (Kupffer cells), adrenal, and hypophysis are frequently grouped together under the term littoral cells or special endothelium. They are sharply differentiated from ordinary endothelial cells (category 1 in Table I) which have practically no phagocytic and greatly restricted developmental potencies.

Much confusion exists as to the relationships, developmental potencies and classification of the free cells in Table I. Opinion varies most with regard to the classification and developmental potencies of the lymphoid cells. They occur in the reticular tissues in three main sizes, i.e., small, medium, and large. (The medium tissue lymphocyte is identical with the large lymphocyte of the blood, and the large tissue one does not usually occur in the blood.) Large lymphocytes are intensely basophil. All three types, together with the hemocytoblast, the equivalent of the large lymphocyte in myeloid tissue, are included under lymphocyte or lymphoid cells, in this review. Nobody doubts that some of these cells, under proper stimuli, have considerable mesenchymal potencies. Many, however, deny that any lymphoid cell with such potencies is a lymphocyte but would classify it as a monoblast, lymphoblast, myeloblast, etc., depending upon the particular theory of hemo-

poiesis held [cf. Osgood (13)]. For example, the large basophil lymphoid cell, which shows a marked reactivity in infections, immunization and *in vivo* immune reactions, has been termed variously a large lymphocyte (14, 15, 16), an acute splenic tumor cell (17), a myeloblast (18), a "lymphoblastic" plasma cell (19), a developing (20) or immature plasma cell (21), a macrohistiocyte (22), and a basophil macrophage (23).

Monocyte is used in this review in the contemporary hematological sense for the large mononuclear and transitional leucocytes of Ehrlich (not for macrophages or the entire mononuclear inflammatory exudate cell series as used by some). In this sense, the monocyte, although differentiated in the phagocytic direction, has closer affinities to the lymphocyte than to the macrophage [Maximow (5, 6)].

There is general agreement regarding the origin and behavior of the granulocytes (2, 5, 6). Heterophils (polymorphonuclears, neutrophils, pseudoeosinophils, etc.) will be considered with the other phagocytes. Eosinophils are less phagocytic than heterophils [Hertzog (24), Ingraham & Wartman (25)]. They were classified by Metchnikoff (26) as microphages. They usually appear late in chronic infections and promptly when parasites are introduced into immune animals. Because they are found locally or generally in large numbers in various hypersensitive states, such as anaphylaxis, serum sickness, bronchial asthma, and helminth infections, they are presumably associated with antigen-antibody reactions [cf. Campbell (27)]. Working with ascaris keratin, Campbell (28) reported that the degree of local eosinophilia which invariably accompanies local anaphylaxis is more closely associated with the shock syndrome than with the primary antigen-antibody reaction and that a lack of eosinophil response in sensitized animals after repeated injections is due to the neutralization of the hypersensitive state and not to a depletion of eosinophil reserves. Reviews of other work may be found in Ringoen (29) and Rebuck (2).

Little is known regarding the basophil [Michels (30), Plimpton (31)]. It is probably different from the connective tissue basophil [review in (2)] which is a source of heparin according to Holmgren & Wilander and Jorpes [review in Ehrich *et al.* (32)] and may have unexplored importance in inflammation.

Many of the basic ideas of the functions of the cells in immunity have originated from a study of their behavior *in vivo* during in-

fections and various types of immune reactions. In such studies, it is imperative to obtain tissues from animals early in the infection and at closely spaced intervals rather than to rely on late stages or necropsy material where the body defenses are overwhelmed or pathologic processes are far advanced [cf. discussions in Taliaferro & Mulligan (14), Conway (16), Loosli (33)].

Work has usually centered around the phagocytic cells, i.e., heterophils, monocytes and macrophages, but a large mass of evidence indicates that lymphoid cells are equally involved in immunity. This evidence has been obtained from studying the behavior of lymphocytes in the field of inflammation, in infectious diseases [cf. for example, Taliaferro & Klüver (34), Severens, Roberts & Card (35), Rich (36)], in resistance to tumors and tissue transplants [review in Loeb (37)], and after the administration of lymphocytocidal agents during immunity and antibody formation (see p. 180). Of immediate interest to the subject under consideration is the correlation that has been noted by many investigators between lymphocyte proliferation and immune reactions. It is particularly well illustrated by the closely spaced observations of Taliaferro and his associates on several species of malaria during initial infection and superinfection after the onset of acquired immunity [review in (3, 14); see also McGhee's studies (38) in embryos], by the studies of Conway (16, 39, 40) on the correlation of changes in lymphoid tissue and immunity to *Listeria monocytogenes*, and by the findings of Ehrich & Harris (41) in the popliteal lymph node during antibody formation. The importance of the lymphocyte in producing new phagocytes and in producing and transporting antibody will be considered later (pp. 166 and 181, respectively).

In addition, there is evidence that living lymphocytes have some protective power which may be of value in nonspecific or innate immunity. Thus, Moor & Newport (42) found that the toxicity of several bacterial toxins is reduced by a preliminary incubation with suspensions containing 90 to 95 per cent living lymphocytes. Turley & Dougherty (43) reported a protection against infections of pneumococcus III and IV in mice using lymphocytes from human tonsils and (44) against *Mycobacterium tuberculosis* in guinea pigs using cells from a week-old peritoneal exudate of rabbits. In the latter study, many of the cells were probably large polyblasts and macrophages. Following treatment

with adrenal cortical extract before and after infection, guinea pigs are more resistant to *Clostridium welchi* [Kepl *et al.* (45)] and mice to Type 1 pneumococci [Vollmer & Gillmore (46)]. These findings may be due to the dissolution of lymphocytes and the release of nonspecific protective factors in view of White & Dougherty's results (reviewed on p. 184).

Systems of Connective Tissue Cells

Fruitful generalizations have centered around Metchnikoff's (26) original concept of "systems" of fundamentally phagocytic cells involved in inflammation and immunity (Table I). The best known of these is Aschoff's (8) reticulo-endothelial system (R.E.S.) which was largely based on studies of vital staining and the storage of colloidal dyes by Renaut, Maximow, Goldman, Tschaschin, Kiyono, Aschoff, Downey, Evans and Schulemann. In the restricted sense, it consists of reticular and littoral cells. In the broad sense, it includes macrophages as indicated in Table I.

The monocyte has been erroneously included in this system. In the first place, much recent literature contains the misconception that Aschoff himself included the monocyte in the R.E.S. Although one of Aschoff's categories was "die Splenocyten [a term frequently used as a designation for monocytes] und farbstoffspeichernden Monocyten (Endothelioleukocyten, Bluthistiocyten), welche von den Histiocyten [Table I, histiocytes of ordinary connective tissue] und den Reticuloendothelien [Table I, group 2 under macrophages] ihren Ursprung nehmen," he was not referring to the monocyte of contemporary hematologists. Thus, he identified his splenocytes and "monocytes" as dye-storing histiocytes or macrophages in agreement with Kiyono and specifically excluded from his system the "transitional forms" and "large mononuclears" of Ehrlich which together comprise the monocyte of contemporary hematologists. In the second place, some recent workers have added the monocyte to the reticulo-endothelial system because they believe it is a functional variant of the macrophage [see page 162 and cf. Bloom (47)].

Metchnikoff's macrophage system (26), even though it was worked out before adequate staining methods were available, undoubtedly included all macrophages, intermediate polyblasts and monocytes (Table I). In addition, Metchnikoff considered the glial phagocytes of the brain to be true macrophages, a conclusion

with which some modern workers agree [Dunning & Furth (48), Dougherty (49)]. He held that small lymphocytes are never phagocytic as such, but become so after acquiring an ample layer of cytoplasm. These latter cells, he further stated, were included among the lymphocytes by Ehrlich, were classified as large mononuclear cells by others, and were called by him hemomacrophages and lymphomacrophages. They are probably equivalent to the monocyte and intermediate polyblast as used in this review.

Taliaferro & Mulligan (3, 14), because of the important role of lymphoid hyperplasia in the development of new macrophages in immunity, combine the lymphoid and macrophage systems under the designation, lymphoid-macrophage system (Table I). This system, therefore, includes all macrophages, nongranular leucocytes and various intermediate cells. It has much in common with and includes the "activated" mononuclear exudate cells in inflammation which Maximow (5, 6), beginning in 1902, designated as polyblasts.

Finally, mention should be made of the Gefässwandzellen of Herzog [Marchand (4)] from which many German authors derive inflammatory exudate cells. These cells consist not only of ordinary endothelial cells but also of perivascular histiocytes, pericytes of Maximow, Rouget cells and possibly others. In the last analysis, they are all supposed to be derived from endothelium [cf. Sabin *et al.* (50)]. This concept seems unwarranted because, although endothelial cells of the early embryo have mesenchymal potencies, those of the adult animal, according to most workers, are highly differentiated elements (47).

Mesenchymal Reserves in Immunity

Mesenchymal reserves are of critical importance because neither the predominantly fixed nor mobile cells (Table I) are adequate for any but the mildest defense reactions without calling on the mesenchymal reserves. Phagocytic reserves are particularly important in the nonimmune host because fewer phagocytes are necessary to suppress the infection after opsonins are acquired [cf. Saslaw & Doan (51)]. Mesenchymal reserves in the adult organism [Bloom (52)] give sources for (*a*) red cells; (*b*) white cells; (*c*) phagocytes; and (*d*) connective tissue cells for mechanical support and repair. Furthermore, cells which retain mesenchymal potencies consist predominantly of (*a*) fixed cells, especially the

macrophages marked with an asterisk in Table I (53) and (*b*) free cells which are represented by lymphocytes and hemocytoblasts in Table I, but which may be assigned other names depending upon the particular theory of hemopoieses followed by a given author (47). Immune reactions are associated with marked increases in macrophages, lymphocytes, monocytes, and granulocytes.

Most authors are in accord regarding the developmental potencies of the fixed cells. Some fixed cells can develop into any other connective tissue cell under the proper stimulus. All are usually conceded the power to develop into fibroblasts although some workers believe that the spindle cell of granulation tissue, which often arises from the macrophage and which is ordinarily classified as a fibroblast, is actually a macrophage [cf. Tilden & Arnold (54)]. All have the power of mitotic proliferation and, thus, according to most investigators, account for the increase in number of macrophages in immune reactions (R.E.S. hyperplasia). An increasing number of investigators, however, believe this to be a lesser source and hold that the majority of new macrophages is derived from the mitotic proliferation of lymphoid cells which then develop into macrophages (55). Furthermore, they believe that initial or mild stimuli generally cause a proliferation and heteroplastic development of free cells, that only intense stimuli cause the fixed cells to react markedly and that then the latter frequently respond by initially transforming into free cells [see discussions in Taliaferro & Mulligan (14), Bloom (47), Conway (16)]. This sequence, including the immediate response of free lymphoid cells and a delayed response of fixed cells, is ordinarily accepted for the development of myeloid elements.

There is general agreement that the free mesenchymal reserves consist of free stem cells of the bone marrow (classified by various investigators as lymphocytes, hemocytoblasts, etc.) which can proliferate and develop heteroplastically into various myeloid cells. To these, most workers would add some lymphoid cells of the lymphatic tissues (called monoblast, etc., according to their developmental potencies). Other workers would add all lymphocytes of the lymphatic tissues. They believe that such cells have retained mesenchymal potencies which are realized if sufficiently stimulated (Table I). The heteroplastic development of macrophages from lymphocytes and monocytes in tissue culture, local inflammation, infection and immunization is substantiated by an

impressive series of studies [Kolouch (56), Rebuck (2)], beginning with those of Maximow in 1902 [5, 6, review in (47)]. A few will be reviewed because they involve the study of tissues taken from standardized experimental infections at early and frequent intervals.

In the lungs of dogs with experimental pneumococcus pneumonia, Loosli (33) found that macrophages which replace heterophils and which play an important part in acquired immunity arise chiefly from hematogeneous lymphocytes and monocytes. The alveoli are particularly well suited for such a study because they contain virtually acellular areas adjacent to blood vessels in which the development of undoubted emigrated hematogeneous cells can be followed.

In malaria, Taliaferro & Cannon (57) found that lymphoid hyperplasia occurs chiefly in the spleen and to a less extent in the bone marrow. Taliaferro & Mulligan (14) further found that lymphoid hyperplasia of the spleen consists of an initial rapid mitotic proliferation of medium lymphocytes, a migration of these cells to the red pulp and their transformation into macrophages. In recovering infections, lymphopoiesis causes a marked lymphoid hyperplasia in the spleen. In fatal infections the spleen contains only a few lymphocytes but normal appearing macrophages.

Conway (40) found that, within a few hours after a single injection of *Listeria monocytogenes*, lymphocytes move from lymphatic organs into the blood and lymph streams, and that thereafter transitional stages between lymphocytes and monocytes (monocytoid lymphocytes) appear and a marked monocytosis occurs in the blood. Later, monocytes in the lymphatic tissues develop into macrophages. Conway's investigations (16, 39, 40), especially in nonimmune animals in which the drain on the lymphatic organs is severe, give a remakable picture of the dynamic equilibrium between lymphopoiesis and the development of monocytes and phagocytes and the resultant lability of the lymphatic organs due to the interplay of lymphopoiesis and lymphocyte depletion.

The ability of the lymphocyte to develop into a monocyte is probably the most controversial subject in modern hematology. Practically all investigators agree that monocytes can develop into macrophages (47), but many deny that monocytes can develop

from lymphocytes. Monocytes are supposed to arise from one or several of the following: (*a*) ordinary endothelial cells; (*b*) fixed mesenchymal cells; and (*c*) lymphoid cells (47). Descriptions of the first derivation are not convincing. The second origin is probable, but the process has not been satisfactorily shown except when the fixed cell develops first into a lymphoid cell and later into a monocyte. The third possibility, variously modified, is the most popular one. Thus, lymphoid precursors of the monocyte, to some as to the reviewer, are identical with lymphocytes of the lymphatic tissue and hemocytoblasts of the myeloid tissues, whereas they are entirely different to others and are given other names, the most common of which is a monoblast.

At the present time, it is impossible to resolve the differences between careful workers who have followed the development of cells usually classified as lymphocytes into monocytes and eventually into macrophages [cf. Harper (58), De Bruyn (59), Rebuck (60)] and those who have failed [cf. Hall & Furth (61), Ebert, Sanders & Florey (62)]. It seems likely that specific conditions will be found which stimulate the lymphocyte to develop into monocytes. Otherwise, there is no explanation for the transitional cells between lymphocytes and monocytes (monocytoid lymphocytes) and the disparity between the small number of emigrated hematogenous monocytes and the large number of developing macrophages seen in inflammation and tissue culture by many investigators.

Mesenchymal reserves are probably an important factor in the genetic resistance of mice to *Salmonella typhi-murium* [Gowen & Calhoun (63), Oakberg (64)] and of chickens to pullorum disease [Severens, Roberts & Card (35)].

Localization, Phagocytosis, and Encapsulation

The eventual disposal of invading organisms or other antigenic materials undoubtedly involves phagocytosis and intracellular digestion and is also probably associated with extracellular digestion by enzymes secreted by the phagocytes. Encapsulation with the formation of granulomas and at times fibrotic walls occurs, but is frequently a temporary expedient which is followed by phagocytosis. Although phagocytosis and encapsulation may be primary localizing factors, they are supplemented by nonspecific

factors of inflammation or inflammatory-like reactions and by specific antibody in immune animals.

Biochemical factors involved in inflammation per se have been especially studied by Menkin [review in (65, 66, 67)]. So far, six materials have been isolated from inflammatory exudates. Four of them may have an immediate importance in immunity. They are: (*a*) leucotaxine, a crystalline-like nitrogenous substance, which increases capillary permeability and causes early heterophil migration [cf. also chemotactic factors, McCutcheon (68)]; (*b*) a leucocytosis-promoting factor, a relatively simple polypeptide associated with the pseudoglobulin fraction of exudate [Menkin (69)], which may cause a hyperplasia of granulocyte precursors in the bone marrow and a rise in circulating granulocytes with a shift to the left; (*c*) a leucopenic factor, probably a polypeptide, which may trap leucocytes in the alveolar walls of the lung, the sinusoids of the liver and sinuses of the spleen [Menkin (70, 71)]; and (*d*) necrosin, a highly toxic substance associated with the euglobulin fraction of exudate. Furthermore, the cells themselves during inflammation in different species may differ in their speed of development. Thus, Taliaferro & Bloom (72) found during inflammatory responses to trypan-blue that hematogenous cells in the monkey after 6 hours and in the canary after 3 hours resemble those in the rat after 30 hours as described by Maximow. Analogously, the transition of macrophages into fibroblasts was similar at 18 days in the rat, 7 days in the monkey and 2 days in the canary.

The primary fixation of microorganisms or antigens is ascribed by Menkin (66, 73) to mechanical factors, such as a fibrinous network in edematous tissue and the occlusion of lymphatics by thrombi in which necrosin may be involved. Antibodies, according to him, play an important but secondary role. Menkin's work indicates that these factors can adequately localize colloidal dyes, India ink, and comparatively avirulent organisms. Ascoli (74) has developed somewhat similar ideas under the term of anachoresis, i.e., the ability of tuberculous nodules to attract from the blood and fix various organisms, toxins, etc. The preponderance of opinion, however, indicates that virulent organisms are little affected by these nonspecific factors and that primary fixation by specific antibodies is necessary.

Investigations by a series of workers [review in Cannon (75), Rich (36, 76)] indicate (*a*) that antibody acting as a precipitin or agglutinin and also as an opsonin localizes and fixes invading organisms and soluble antigens in the immune animal and (*b*) that even intense inflammation, such as that following sensitization to an unrelated antigen, does not protect against virulent organisms [Cannon & Hartley (77)]. There is a striking similarity in the fixation of different types of invaders, such as localization and phagocytosis of various bacteria in the skin [Meyer *et al.* (78); and reviews by Cannon (75), Rich (36)], agglutination and phagocytosis of red cells parasitized by plasmodia in the spleen [Taliaferro & Cannon (57)], and immobilization, encapsulation and eventual removal of the nematode, *Nippostrongylus*, in the skin and lungs of immune rats [Taliaferro & Sarles (79)] and of passively immunized rats [Taliaferro & Sarles (80)].

Comparative Importance of Heterophils and Macrophages in Phagocytosis

The facts pertinent to a discussion of the relative importance of the heterophil and macrophage (including one of its precursors, the monocyte) in immunity are these: the heterophil is a short-lived cell, whereas the macrophage is long-lived, can proliferate, and possesses varying mesenchymal potencies. In addition, although the two phagocytes probably have fundamentally different tropisms [Lasfargues (81)], recent studies tend to emphasize the greater importance of the macrophage than of the heterophil in phagocytosis no matter which of them responds most actively at first. In such conditions as malaria (14), worm infections (79, 82), infectious mononucleosis (16) and tuberculosis (36), heterophils are at best transiently involved and give little histological evidence of activity. In bacterial infections in which both heterophils and macrophages ingest large numbers of organisms, it is difficult to assess the importance of the change from heterophil to macrophage exudate [cf. Menkin (65) for a discussion of the basis for this cell succession] because antibodies often rise in titer as macrophages begin to predominate. Nevertheless, several lines of evidence indicate the superiority of the macrophage [reviews in Gay (83), Robertson (84)]. Macrophages digest Type 1 pneumococci more rapidly than heterophils both *in vitro* and *in vivo* although

they are only slightly more phagocytic [Robertson & Van Sant (85)].

The relative ability of heterophils and macrophages to ingest and to digest organisms may vary with the developmental stage of the organism and its location in the host. Thus, macrophages form a suitable medium for the growth of exoerythrocytic stages of plasmodia although they readily digest erythrocytic stages [Huff & Coulston (86)]. On the other hand, while heterophils rarely ingest entire erythrocytic stages in the spleen and liver, they readily do so in the skin [Taliaferro & Bloom (72)].

It has long been known that phagocytes sometimes ingest organisms that they cannot digest and so become vehicles of dissemination [Goodpasture & Anderson (87), Huff & Coulston (86), Taliaferro (55)]. Recently, Merling (88) found that intraleucocytic vaccinia virus forms colonies which survive the leucocyte.

Nonspecific Factors in Phagocytosis

Opsonins are unquestionably the most important factor in the increased phagocytosis of acquired immunity, but are not considered in the present review (cf. 36, 84). There are, however, many nonspecific factors which may operate during both innate and acquired immunity. Mudd *et al.* (89) have reviewed the older work. Recent publications of several types are of importance.

In the living animal, Knisely *et al.* (90) have observed that India ink, kaolin, and graphite particles are removed from the blood by Kupffer cells only if they first acquire a fibrin-like coat. Somewhat similarly, they (91) found in rhesus monkeys that red cells shortly after penetration by *Plasmodium knowlesi* become coated with a thin layer of precipitate, which makes them adhere to other similarly coated parasitized red cells but not to normal cells and results in their being selectively phagocytosed by the Kupffer cells. Later in the infection, fluffy precipitate forms around and between all blood cells, whether normal or parasitized, and changes the blood to a thick sludge-like consistency. In this last stage, phagocytosis is greatly reduced because, according to them, the flow of blood through the sinusoids is decreased and the phagocytes are unable to ingest such large masses. The extent to which these precipitates in malarious animals may be due to antibody is not known.

Other nonantibody factors may play a role in phagocytosis as indicated by the studies of Harmon *et al.* (92) on temperature relations in the phagocytosis of staphylococcus *in vitro*, of Redmond (93) on the electric charge of red blood cells in malaria (although it may be secondary to antibody action), and of Nungester & Ames (94) on ascorbic acid content of exudate in relation to *in vitro* heterophil fragility and the phagocytosis of beta hemolytic streptococci.

There are striking inabilities to demonstrate antibodies in some infections in which recovery simulates or is due to acquired immunity. In part, these failures may be due to technical difficulties associated with the demonstration of low concentrations of antibody in the blood although sufficient may occur at the site of the immune reaction [see discussion of malarias by Taliaferro (95)], to the protection of the parasite by the host cell as, for example, in various plasmodia [Coggeshall (96)] and in intracellular viruses [Rivers (97)], or to the antibody being an immune isoopsonin to the host cell as in malaria [Zuckerman (98)]. In part, however, nonspecific factors may operate. Where antibodies are demonstrated by protective tests, there may be an inadequate nonspecific activation or mobilization of cells of the lymphoid-macrophage system of the recipient animal [Gay *et al.* (83), Webb (99), Mulligan *et al.* (100)]. In some malarias, Rigdon & McCain (101) believe that parasites die because of a decrease in mature red cells, which are preferred by the parasite, and an increase in carbon dioxide content of the plasma.

There is excellent evidence for believing that an important factor in tuberculosis immunity is a decreased survival of organisms within macrophages [review in Rich (36)]. Lurie (102) believes that this is largely associated with a nonspecific physiological activation as indicated by an increased phagocytic activity against unrelated particulate matter, such as carbon and colloidion particles [cf. the parallel increased cathepsin hydrolysis by Weiss & Halliday (103), and the disappearance of leprosy bacilli from fibroblasts cultivated from lepromas by Hanks (104, 105)]. Lurie (106) allowed macrophages derived from normal or immunized animals to ingest tubercle bacilli with and without immune serum and then transplanted them to the anterior chambers of normal rabbit eyes for further study in the absence of antibodies. He concluded that active infection confers upon the

macrophages bacteriostatic properties which are independent of immune body fluids [cf. Rich (36)]. Kallós and his co-workers (107) reached somewhat similar conclusions, but place more emphasis on the ablastic action of humoral principles.

In experimental pneumonia produced by the pneumococcus or Friedländer's bacillus, Wood and his co-workers (108, 109) ascertained that phagocytosis of pneumococci in rats in the absence of antibody is largely dependent on the type of surface upon which the macrophages move. Thus, phagocytosis occurs in the lungs of rats without antibody, in formalin-fixed lungs *in vitro*, and on the surfaces of moistened filter paper, cloth and fiber glass, but not on smooth materials, such as glass, cellophane, albumin, or paraffin, whereas antibody-induced phagocytosis occurs on all these surfaces. Digestion is equally rapid in both cases. He termed the nonantibody phagocytosis, surface phagocytosis [cf. Ørskov (110)].

Factors Injuring the Phagocytic Mechanism

It has long been known that reticulo-endothelial blockade, splenectomy and lymphocytocidal agents, such as x-rays, the sulfur and nitrogen mustards, and benzol, lower phagocytic immunities. Recent papers contain studies of R.E.S. blockade in blastomycosis (111) and malaria (112); splenectomy in malaria (113 to 116) and tapeworm infections (117); x-irradiation in bacterial infections of the skin (118) and in protozoan infections (119, 120, 121); and nitrogen mustard in malaria (122). The results with these experimental procedures have to be interpreted with regard to such factors as species differences in the size of the spleen, stimulating effects of small doses of blockading agents, physiological compensation [cf. Gabé (123)], virulence of the organism or degree of immunity present (121, 122), and parasiticidal effects of injurious agents (122). A discussion of such factors in relation to the R.E.S. is given by Jaffé (11).

The effect of such procedures, when they involve acquired immunity, is probably largely to depress antibody formation (see p. 180). Furthermore, in malaria, where it is possible to differentiate reproduction-inhibiting from parasiticidal factors in immunity, R.E.S. blockade reveals that phagocytosis of the parasites by macrophages is fundamentally different during innate immunity than after the supervention of acquired immunity. Thus, Ging-

rich's (124) work suggests that parasites phagocytosed in the animal during innate immunity would have died anyway. Working with infections of *Plasmodium cathemerium*, he found that extensive blockade with foreign red cells does not influence the death rate of parasites in animals with innate immunity, but markedly lowers it after acquired immunity has supervened. He interpreted these results to indicate that phagocytosis, whatever its relation to humoral factors may be, is a primary factor in acquired but not in innate immunity. Similarly, splenectomy (114, 115, 116) and nitrogen mustard (122) lower acquired but not innate immunity in chicken malaria.

Since protein deficiency is associated with a lowered power to synthesize and maintain antibody levels [review in Cannon (125, 126)], it undoubtedly lowers immune opsonins. In addition, protein as well as other dietary deficiencies lower the efficiency of the innate phagocytic mechanism by decreasing the phagocytic activity of leucocytes and by depleting the mesenchymal reserves.

According to Cottingham & Mills (127, 128), heterophils in heparinized whole blood of protein-deficient rats or of rats with growth-retarding deficiencies of thiamine, riboflavin, pyridoxine, pantothenic acid, choline or ascorbic acid show a lower phagocytic index for *Micrococcus candidus* than do those from adequately fed animals. Phagocytosis is mildly reduced during deficiencies of vitamin A and D but not of inositol and *p*-aminobenzoic acid, and seems most efficient with vitamin intakes higher than those needed for optimal growth. Cottingham & Mills (129) also found that rats on deficient diets begin to lose weight during the first week, but show a reduction in phagocytic activity only from the second through the fourth week. Using the same methods, Berry *et al.* (130) reported that rats deficient in proteins, vitamins and mineral supplements develop a leucopenia with a 60 to 65 per cent reduction in activity of heterophils, although these cells show a relative increase in numbers.

The use of nonpathogenic *M. candidus* to test the phagocytic index of heterophils by Cottingham & Mills and Berry *et al.* makes the presence of natural antibodies unlikely, but their use of whole blood makes it impossible to evaluate the role of nonspecific humoral factors. That such may be important is indicated by the earlier work of Gellhorn & Dunn (131) who found that serum from rats after prolonged vitamin A deprivation may lose

much of its power to stimulate the phagocytosis of starch granules by heterophils. Furthermore, Guggenheim & Buechler (132) ascertained that the humoral defense mechanism is more sensitive than the cellular mechanism to protein depletion as tested by the disappearance and phagocytosis of bacteria in the peritoneal cavity of unimmunized rats.

Berry *et al.* (133, 134) reported marked increases in the phagocytic activity of heterophils for *M. candidus* in anemic patients even when malnourished. These increases are associated with a change in the leucocytes rather than in the serum. Berry & Haller (135, 136) also produced a similar increase in phagocytic activity in rats (both heterophils and macrophages) and mice made anemic by bleeding, which in the latter animals is associated with an increased resistance to *Salmonella typhi-murium.*

Cannon (126, 137) points out that, after severe protein depletion, mesenchymal reserves may be too depleted to form sufficient phagocytes and heterophils may be too immature to possess full phagocytic powers. Kornberg (138) demonstrated the inability of rats to form granulocytes unless furnished the proper amino acids in the diet.

Failure of the defense mechanism in rabbits and rats against pneumococcus infection following protein depletion is largely due to a decrease in antibody formation but is probably partly due to an impaired functional ability and a lessened production of leucocytes, according to Wissler (139, 140). In studying the inflammatory response to the subcutaneous injection of turpentine, Asirvadham (141) observed that the leucocytic response is greatly impaired in protein-depleted rats, especially after repeated stimulation, with a significant reduction in circulating lymphocytes but not of heterophils (cf. 130). Further analysis indicated no lack of the leucocytosis-promoting factor of Menkin (66) in the inflammatory exudate, or of the chemotactic substances which attract leucocytes to the site of injury, but an inability of the bone marrow to produce leucocytes. There was depletion of both myeloid and lymphoid elements.

During work on the lowering of malarial immunity by x-rays and nitrogen mustard, Taliaferro and his co-workers (121, 122) have stressed the importance of the injury to mesenchymal reserves (lymphoid cells) from which macrophages arise.

The mobilization of markedly immature heterophils in de-

pleted animals is undoubtedly of significance as indicated by the following studies, even though the optimal stage of their activity is not clearly established. Strumia & Boerner (142) and Hertzog (24) found that hemocytoblasts are not phagocytic, but that more immediate heterophil precursors are increasingly so as the cells mature. There is doubt as to whether the band is more active than more mature heterophils (24, 51, 142). As lymphocytes and monocytes develop into macrophages, phagocytic activity is directly correlated with size of the cell, as first noted by Metchnikoff and described in detail by others [Taliaferro & Cannon (57), Hertzog (24), Rebuck (2)]. In later stages of inflammation and in tissue culture, phagocytic activity rapidly declines and eventually disappears as macrophages develop into fibroblasts.

Effective phagocytosis forms an interesting contrast to the successful maintenance of leishmania in macrophages. Ritterson & Stauber (143) found that leishmaniasis leads to earlier emaciation and death of hamsters on protein-deficient diets than of normal animals. Further analysis indicated that normal and depleted animals contain the same number of parasites in their spleens but that normal animals have a marked splenomegaly and, hence, fewer parasites per macrophage. They believe that reproduction of parasites in the normal animal produces a progressive functional blockade of the R.E.S. which is matched by a compensatory hyperplasia of macrophages.

Adequate antibody and phagocytic reserves may be neutralized by certain poisons. Thus, Lushbaugh (144) observed that rabbits immune to intradermally injected virulent pneumococci often suffer a severe and sometimes fatal bacteremia after alcoholic intoxication. This breakdown of the defense mechanism is associated with a sluggish inflammatory response and a delayed mobilization of phagocytes at the site of inoculation.

The Role of Complement in Phagocytosis

Fresh normal serums from various species, according to Ecker and his associates (145, 146), possess thermolabile opsonic powers which do not parallel hemolytic complement. The thermolabile opsonic property in normal human serum against the nonpathogenic *Micrococcus candidus* is identical with a combination of C′1, C′2, and C′4 components. None of these components alone or combined with only one other is effective (147).

The role of complement in antibody-induced phagocytosis was also studied by Ecker *et al.* (145). These investigators found little phagocytosis of staphylococcus by heterophils in the absence of serum. Inactivated immune opsonic serum exhibits a marked zone phenomenon. Addition of fresh guinea pig serum to optimal or suboptimal concentrations of immune serum does not enhance but depresses phagocytosis to about the range of fresh guinea pig serum alone. Ecker and his associates recognized the large number of variables in carrying out opsonic tests *in vitro* and took unusual care to control them. Their results are not always in agreement with past work, possibly due to the use of different organisms or different techniques. For example, Welch *et al.* (148) corroborated the earlier findings of Ward & Enders to the effect that complement does not increase the peak amount of phagocytosis of virulent pneumococcus in the presence of immune serum but accelerates the rate at which the peak is reached. On the other hand, Maaløe (149) reported that complement (in a concentration above 1:1000) must be present with antibody to induce phagocytosis of *Salmonella breslau.* If corroborated, the activity of complement in such high dilutions would be unique.

In addition to the role of complement in phagocytosis, Delaunay & Pagès (150) found that complement is necessary for chemotaxis and stimulates the respiratory metabolism of heterophils.

Cellular Factors in Chemotherapy

Successful drug treatment of infections, in addition to the direct action of the drug in microbial metabolism, is undoubtedly associated with the defense mechanisms of the host. Some investigators believe the defense mechanisms to be an integral part of the chemotherapeutic mechanism, whereas others find them to function fundamentally in an independent coordinate manner [cf. Maher (151), for a comprehensive review on bacterial chemotherapy with the sulfonamides, and Taliaferro *et al.* (115, 116, 152) on protozoan infections].

Phagocytosis frequently accompanies drug action [Tunnicliff (153), Lushbaugh & Cannon (154), Haag (155), Wood & Irons (156), Wood *et al.* (157), Sale *et al.* (158), Skinsnes (159)]. That the host cell may be acted upon is indicated by the observation that sulfonamides stimulate phagocytosis of streptococci *in vitro*

by acting on the heterophils rather than by acting on the bacteria in an opsonin-like capacity [Tunnicliff (153, 160)] and by the finding that sulfathiazole stimulates phagocytosis of trypan blue [Borell & Troell (161)]. In other cases, the drug is assumed to act primarily (*a*) by inhibiting microbial metabolism to such an extent that the parasite no longer produces substances which prevent the migration and mobilization of leucocytes in the tissues [Kollath & Raabe (162)] or (*b*) by making microorganisms more phagocytable [Leonard (163) and others]. Dubois & Kohn (164) question the primary importance of phagocytosis because they found that trypanosomes removed from a host shortly after treatment and injected into splenectomized and blockaded animals are removed just as fast as when injected into intact animals. They believe that the parasites are so injured by the drug that they perish with or without the presence of functional phagocytes (cf. 152). On the other hand, Wood and his associates reported that nonantibody surface phagocytosis is important in rats treated with sulfonamides during recovery from experimental pneumonia produced by pneumococcus (156, 165) and Friedländer's bacillus (109, 158). Others stress the independent action of drugs and immunity [Harris & Miller (166) and Lushbaugh & Cannon (154) with sulfonamides, and Harrison (167) with penicillin and sulfonamides].

Skinsnes (159) studied penicillin treatment in intradermally-induced pneumococcus infection in rats and guinea pigs with the following results: The antibiotic does not stimulate phagocytosis of the organisms. The defense mechanisms of the host play a minor role in early adequately treated infections but become progressively more essential with the postponement of treatment. Treatment was also studied in protein-depleted rats, which exhibit a marked decrease in phagocytic ability, and in nitrogen mustard-treated guinea pigs, which exhibit a leucopenia and a markedly impaired general defense reaction. The impairment is not a handicap in adequate early treatment, but, when treatment is delayed, necessitates prolonged treatment.

The role of the host during the quinine treatment of malaria has been investigated by Taliaferro *et al.* (115, 116, 152). Their observations and results may be summarized as follows: Quinine is not converted by the R.E.S. into a more active product nor does it make the parasites more vulnerable to phagocytosis al-

though it markedly inhibits their reproduction. The spleen plays two antagonistic roles during treatment. It decreases the suppressive action of quinine by reducing the blood concentration of the drug, i.e., the drug parasite contact, and increases the suppressive effect of acquired (but not innate) immunity. Acquired immunity, although auxiliary to quinine, is highly important in supplementing the antimalarial action of quinine, especially after discontinuance of the drug. Three independent antimalarial factors operate during the quinine treatment of malaria, viz., (*a*) innate immunity, (*b*) acquired immunity, and (*c*) quinine.

Site of Antibody Formation

There is conclusive evidence that antibodies are formed in reticular tissues and strong evidence that they may be formed in granulomatous nodules in the skin. Evidence is incomplete as to what tissues or cells cannot form them and occasionally authors believe that an essential part of their synthesis occurs in the blood [Bacon (168)]. The outstanding contribution of the older literature is the demonstration that extirpation of the spleen frequently lowers acquired immunity including the formation of specific antibodies (9, 10, 11).

That antibodies are formed in individual lymph nodes was first unequivocally demonstrated by McMaster & Hudack (169) with various bacterial antigens and by McMaster & Kidd (170) with vaccinia virus. These results were corroborated and extended by Ehrich & Harris (41), who injected typhoid vaccine, sheep erythrocytes, and egg albumen into the hind foot of the rabbit. With this procedure, antibodies appear in the efferent lymph of the popliteal node two to four days after injection of antigen, reach their highest titer after six days and are always higher, sometimes as much as 100 times higher, than in the afferent lymph.

The local production of antibodies is difficult to differentiate from the local concentration of antibodies (formed elsewhere) due to the increased capillary permeability associated with inflammation [Fox (171), Menkin (65), Rigdon (172)]. Cannon and his associates [review in Hartley (173)] have found evidence for the local production of antibodies. The latter investigator, by injecting aluminum hydroxide gel into the skin of rabbits 18 and 21 days previously, produced nodules consisting predominantly of macrophages with a few heterophils and lymphocytes and a

minimal increase in fibroblasts. Neutralizing antibodies often appear in such areas following local injection of vaccinia virus adsorbed on aluminum hydroxide gel before they do in other tissues or the blood, and reach a higher titer. Concentration due to local inflammation is not a major factor because antibodies do not reach as high a titer in such areas as in the blood when the virus is injected intravenously.

Many workers have found that the formation of specific antibodies decreases after (*a*) reticulo-endothelial blockade; and (*b*) injury to various lymphoid cells by x-rays or other ionizing radiations, the nitrogen and sulphur mustards, and benzol. Recent work includes x-rays [Naiman (119), Fredell *et al.* (174)] and nitrogen mustards [Philips *et al.* (175), Spurr (176)]. Also, it should be pointed out that pyridoxine deficiency produces a marked loss of thymic and lymphoid tissue and is associated with a lowering of antibody formation [Stoerk *et al.* (177)]. All these investigations, although they indicate that macrophages, lymphocytes, and possibly other cells contained in exudates form antibodies, do not definitely differentiate between the cells and are open to the criticism that physiological effects, unrelated to or secondary to the observed histological changes, cannot be ruled out. Although blockading agents are taken up by macrophages, they may cause a secondary depletion of the lymphoid cells of the spleen and bone marrow when injected intravenously. Similarly, lymphocytocidal agents damage lymphoid cells, but macrophages may be more or less temporarily blockaded by ingesting the resulting debris. Moreover, ionizing radiations not only damage lymphocytes in the lymphatic tissues [Murray (178), De Bruyn (179)] but all lymphoid stem cells in the bone marrow [Bloom & Bloom (180, 181)] and various members of the erythrocyte and myelocyte series. Macrophages and reticular cells are resistant. Much the same results are obtained with the mustard gases [Cameron *et al.* (182), Graef *et al.* (183)] and benzol [Selling & Osgood (184)].

Currently, antibody formation can be conveniently separated into the lymphocyte, plasma cell, macrophage, and various combined or multicell theories. These had best be considered with the data on the antibody content of the cells upon which they are based.

Cells Involved in Antibody Formation

Several authors have concluded that antibodies occur in lymphocytes. Dougherty, Chase & White (185) reported that cell suspensions from lymph nodes and thymuses comprising about 90 per cent lymphocytes contain significantly more antibody than serum. In continuing their studies on the occurrence of antibodies in the efferent lymph of the popliteal lymph node after injecting antigen into the rabbit's foot pad, Harris *et al.* (186) found that the cellular fraction of the lymph, which includes about 99 per cent lymphocytes, often contains 8 to 16 times more antibody than the plasma fraction. In view of the fact that normal lymphocytes do not acquire antibody either after incubation for several hours in antibody-containing plasma or in passively immunized animals, they conclude that lymphocytes produce rather than absorb or adsorb antibody.

White & Dougherty (187) leave open the question of the primary site of antibody formation, but point out that labelled globulin in lymphocytes is passed on to daughter cells and must increase in amount as the cells proliferate. Thus, they transplanted antibody-containing malignant lymphocytes and found that tumors developing from the transplants contain as much antibody as the original tissue.

Another line of evidence indicating that exudate cells and especially lymphocytes may contain antibodies involves the passively transferable hypersensitivities. Landsteiner & Chase (188) obtained passive transfer of the delayed type of skin sensitivity to drugs with exudate cells, and Chase (189) of tuberculin sensitivity with peritoneal exudates or cells from the spleen or lymph nodes. Others have verified these findings with general [Kirchheimer & Weiser (190), Cummings *et al.* (191)] and local transfer [Metaxas & Metaxas-Bühler (192)]. All of the transfer materials contained some lymphocytes, and in many instances these were the predominant cell type. Stavitsky (193) obtained the transfer with suspensions containing 95 per cent lymphocytes.

Cytolysis, which needs complement and is exhibited when blood cells from infected hosts are subjected to specific antigens as described by Favour (194) and Fremont-Smith & Favour (195), suggests intracellular or adsorbed antibody. These investigators found that lymphocytes from tuberculous humans,

mice, and guinea pigs exhibit lympholysis when subjected to tuberculoprotein. Heterophils are affected only when a delayed tuberculin-type of skin test occurs (guinea pigs and humans). Lympholysis also occurs when lymphocytes of mice infected with *Salmonella enteritidis* are mixed with enteritidis-soluble substance. These results should be compared with the earlier work on the *in vitro* susceptibility of blood leucocytes, and spleen and bone marrow cells from tuberculous animals to tuberculin but not of anaphylactically sensitive animals to homologous antigen [review in Rich (36, 76)].

Plasma cells, which often appear in large numbers at about the time of the onset of acquired immunity and persist for long periods during chronic infections, were associated with antibody formation as early as 1913 by Heubschmann (19). The earlier work indicating their role in hyperglobinemia and antibody formation is reviewed by Fagraeus (21). Kolouch (196) found that a rise in blood antibody is associated with a transformation of bone marrow reticular cells into plasma cells. Bjørneboe & Gormsen (197) concluded that there is a constant relationship between antibody formation and plasma cell infiltration. With Lundquist (198), they observed that adipose tissue of the renal sinus after immunization has a massive plasma cell infiltration and a greater antibody content than other organs.

Recently, Fagraeus (21, 199), in studying the secondary antibody response in rabbits reinjected with horse serum, egg albumin, azoproteins and *Salmonella typhi*, described (*a*) an increase and localization of plasma cells in the red pulp of the spleen simultaneously with an increase in antibodies, and (*b*) an increase in antibodies in tissue cultures from such localizations. Although the association of plasma cells with immunization and antibody-formation is striking, it is not entirely convincing because of the many variables. Fagraeus (21), however, does not assume that the plasma cell per se is the most important source of antibody. She believes that antibodies are formed by the reticulo-endothelial cells and that intense antibody production leads to the production of plasma cells which are in turn cells that have passed their greatest functional activity. Kolouch *et al.* (20) associate the basophilia of the early plasma cell with protein formation and likely antibody formation [cf. Good (200)].

Both Fagraeus and Kolouch consider the ever-recurring ques-

tion of the classification of the cells involved. They picture transitional stages between reticular cells and plasma cells which they identify (20, 21) with lymphoid cells. To those who consider such cells to be lymphocytes (see p. 162), the data would actually support the lymphocyte theory of antibody formation.

The macrophage theory of antibody formation is based upon the reasonable assumption that cells which remove and digest particulate antigen should also form antibodies. It is supported chiefly by the long series of investigations on R.E.S. blockade previously mentioned (p. 180). A striking presentation of this theory is given by Sabin (201).

Ehrich *et al.* (202) found only low concentrations of agglutinin in exudates from the foot pads of rabbits injected with dysentery vaccine with or without paraffin oil, or from the peritoneal cavity of animals injected intra-abdominally with various dysentery and typhoid antigens, although the general antibody response was excellent with both procedures. Isolated granulocytes and macrophages from the peritoneal cavity yielded no antibody. They believed that the acidophil nature of the cytoplasm of macrophages and heterophils speaks against anabolic phases of globulin synthesis because, where protein is synthesized, the cytoplasm is basophil due to the presence of ribose nucleic acid [cf. the work of Caspersson and others, review in (21, 203)]. Harris & Harris (203) also associated cytoplasmic ribose nucleic acid (pyronine-staining granules) with protein and hence with antibody formation, and localized it chiefly to large lymphocytes and cells transitional between them and reticular cells (see discussion of plasma cells). No increase of plasma cells was observed. On the other hand, Houghton (204) reported that antibodies do not appear in the serum residues of cultures of washed cells from the peritoneal cavity of rabbits (injected for six days with ovalbumin and once with pumice) until the sixth day of incubation, and thereafter run parallel with titers in the immunized animals. When antibodies appear in the cultures, only clasmatocytes (=macrophages) and monocytes or their modified forms are present.

Ehrich & Harris (205, 206) modified an earlier suggestion of Bunting to the effect that heterophils and macrophages destroy living organisms, and lymphocytes form antitoxins. They believe that phagocytes break down corpuscular antigen and prepare it for proper utilization by lymphocytes; that the effect of blockade

on antibody formation is an effect on the preliminary preparation of the antigen; and that the enhancement of immunization by the use of adjuvants, such as those of Freund (207), is brought about by delaying the destruction of antigen by phagocytes to a rate at which it can be advantageously utilized by the lymphocyte [see also (208)]. It has been pointed out against this view, however, that the enzymatic degradation of antigen cannot be carried very far without a complete loss of antigenicity [cf. discussion in (206)]. In support of their view, Harris & Ehrich (209) described the production of soluble antigenic materials from cellular antigens and the probable sequence whereby these materials are made available to the lymphocytes of the regional lymph node.

Hormonal Control of Antibody Release

White & Dougherty and their associates [review in (187, 210, 211)] have published a series of papers on the hormonal control of the synthesis, storage and release of antibodies by lymphocytes. Their experiments and conclusions involve the following considerations: Lymphocytes of normal animals contain a protein identical with serum γ-globulin [cf. Kass (212)] and probably a second one identical with serum β-globulin as determined by Tiselius electrophoretic analysis (187, 213). Hormonal control is important in the release of these globulins because, following pituitary stimulation or the administration of excess adrenotrophin or adrenal cortical preparations, lymphocytes are dissolved in lymphoid tissues and shed their cytoplasm, i.e., release globulin, to the extent that a marked lymphopenia and an increase in serum β- and γ-globulins, but not of α-globulin, occurs in the blood (214, 215, 216). The availability of these proteins during stress is probably contingent on the presence of the pituitary and adrenals, because lymphoid tissue does not show these changes and reserve lymphocyte protein is not released in adrenalectomized animals during a period of fast. The same type of pituitary-adrenal-cortical control exists in immune animals except that some of the lymphocyte protein released by lymphocyte dissolution is antibody. Thus, previously immunized animals with no demonstrable circulating antibody have antibody within their lymphocytes and show an anamnestic response after the administration of pituitary-adrenal-cortical hormones (187, 217, 218). These investigators believe that increased secretions of adrenotrophic hormones may occur

after a variety of stimuli which are known to activate pituitary-adrenal-cortical secretion. Thus, hemorrhage, cold, heat, bacterial toxins, foreign proteins, and toxic chemical agents, by operating through the hormonal system, precipitate anamnestic responses (187). Benzene, arsenite, and low doses of x-rays, such as 10 r in mice, only disrupt lymphocytes and release antibody in the presence of adrenals but high doses of x-rays (200 r) act in adrenalectomized as well as normal animals (219).

The foregoing conclusions have been questioned in part by some recent investigators. Thus, Abrams & Cohen (220) were unable to find γ-globulin in lymphocytes from human tonsils and thymuses of normal calves. Murphy & Sturm (221) reported a lymphoid hyperplasia with a marked increase in antibody formation in adrenalectomized rabbits and concluded that adrenal cortical hormone extract is not necessary for the release of antibodies from lymphocytes. Suppressing lymphoid hyperplasia in adrenalectomized animals by administering adrenal cortical preparations, however, does not suppress antibody formation to the normal level because, according to them, lymphocytes are disrupted by the hormone. Eisen *et al.* (222) questioned the relative significance of the increases in antibody production reported by Chase, White & Dougherty (223) following the simultaneous injection of adrenal cortical extract and antigen. They used quantitative methods of antibody titration and studied the effect of adrenal cortical extract in adrenalectomized animals maintained on sodium chloride and desoxycorticosterone acetate which was reported by Dougherty *et al.* (218) to have no effect on antibody levels. They found identical concentrations of antibodies and γ-globulin in adrenalectomized rats with or without repeated injections of adrenal cortical extract during immunization and conclude that adrenal cortical activity is not essential for the fabrication or release of antibody. They did find in agreement with Chase, White & Dougherty (223) that the administration of adrenal cortical extract to previously immunized animals sometimes results in an approximate 30 per cent increase in antibody N, which lasts only about 12 hours, but believe this to be a nonspecific anamnestic response.

Houghton *et al.* (224) failed to find that adrenal cortical hormones either increase the capacity of animals to form antibodies or alter titers after antibodies are formed. More recently, Fischel

et al. (225) failed to find any rise in circulating antibody (using a quantitative precipitin method) following the administration of adrenocorticotrophic hormones or 50 r x-rays three months after cessation of immunization although a lymphopenia occurred.

In studying the decline of hemolysins (determined photometrically), Taliaferro & Taliaferro (226) failed to find any increase in antibody after irradiation of rabbits with one or two doses of 400 r hard x-rays. Until further evidence is accumulated, it is well to keep in mind that various factors may influence the effect of x-irradiation [cf. Glenn (227, 228)].

Cellular and Protein Reserves

Recent work emphasizes the importance of mesenchymal reserves as a ready source of both connective tissue cells and antibody globulin. In spite of the large number of phagocytic cells available, mesenchymal reserves are usually stimulated to produce, first, phagocytes (and other connective tissue cells) and, later, antibodies. Mesenchymal potencies are admitted for predominantly fixed cells (macrophages) and for at least some lymphoid cells. Opinion varies, however, from the view that all lymphoid cells are lymphocytes and that they possess extensive developmental potencies to the view that no lymphoid cell with these potencies can be considered a lymphocyte. Some macrophages undoubtedly arise by the mitotic division of pre-existing macrophages. Increasing evidence indicates, however, that the development of monocytes and most macrophages, under conditions of stress, from fixed cells involves a lymphocyte-like intermediate step. This is similar to the accepted development of phagocytes and free myeloid elements from fixed reticular cells via a lymphocyte or "blast." In general, however, free cells in both myeloid and lymphoid organs respond first.

Antibodies are undoubtedly formed in the spleen and lymph nodes and probably in granulomatous nodules in the skin. Experimental evidence that antibodies occur in lymphocytes is of greatest importance [see recent work of Harris & Harris (229)] but should be interpreted with due regard to technical difficulties [Habel *et al.* (230)] and is open to the possibility (*a*) that antibodies are produced elsewhere and that lymphocytes absorb them by a dynamic metabolic transfer or adsorb them or (*b*) that a few nonlymphoid cells in the suspensions contain the antibody. This

last seems unlikely since contaminant cells were reduced to 1 per cent by Ehrich and his associates. On the other hand, studies by Hartley (173) and Houghton (204) indicate macrophage participation. There is much to favor the idea that both macrophages and lymphocytes are involved. The fact that a severe drain on the fixed mesenchymal reserves may result in the initial transformation of these fixed cells into large free basophil cells (lymphocytes) would permit the following: (*a*) initial ingestion of antigen by the macrophage with a transfer of specific antibody-producing synthetic enzymes to the large basophil cells would be compatible with many of the ideas of Ehrich and Harris and is similar to the plasma cell theories of Kolouch and Fagraeus; (*b*) the original macrophages or their descendants by mitotic proliferation or by secondary development from intermediate basophil lymphoid cells could directly form antibodies provided protein synthesis is not exclusively associated with marked basophilia of the cytoplasm.

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VIRULENCE AND PATHOGENICITY[1]

By D. W. Watson[2] and C. A. Brandly

Departments of Agricultural Bacteriology and Veterinary Science
University of Wisconsin, Madison 6, Wisconsin

The complex phenomenon encompassing virulence and pathogenicity is difficult to treat as a yearly review. The subject, if defined as an expression or interaction of the host-parasite relationship, has so many components that an adequate survey of all papers pertinent thereto would be impossible within the space allotted. This review will include not only studies dealing with the various factors associated with the parasite as they apparently influence virulence but also those pertaining to the several properties of the host which are equally important for an analysis of the problem. Material has been selected from the various fields of bacteriology and virology to illustrate the general methods and trends in current investigations.

Many phases of the subject have been reviewed in the first and second volumes of the *Annual Review of Microbiology*. Within recent years several reviews have appeared; Dubos (1) in his excellent treatise on the "Bacterial Cell" has presented a lucid chapter on the nature of virulence; the various mechanisms of pathogenicity and virulence have been well presented by Raffel (2). Rich (3) has contributed a clear statement of the nature and effects of virulence as applied to the pathogenesis of tuberculosis. More recently, a general discussion on the properties of bacteria which enable them to cause disease has been written by MacLeod & Pappenheimer (4). During 1946, several workers in England took part in a symposium on the nature of virulence. Unfortunately, the papers and discussions were not published in full, but the abstracts serve to point out many of the problems associated with the virulence of bacteria in plants (5), bacteria in animals (6), in protozoa (7), of fungi in animals (8), of viruses in plants (9), and of viruses in animals (10).

Concepts of Virulence and Pathogenicity

Pathogenicity is usually defined as the ability of a parasite to

[1] This review covers the period up to March, 1949.

[2] Present address: Department of Bacteriology and Immunology, Medical School, University of Minnesota, Minneapolis 14, Minneapolis.

cause disease, either natural or experimental, while the term virulence is reserved to quantitate or express the degree of pathogenicity. The present tendency, however, is to use the terms synonymously or, more frequently, to employ the term virulence as taking into consideration both the qualitative and quantitative factors associated with the capability of a parasite to cause disease. Others would reserve the term pathogenicity for those agents which do not lead to rapidly fatal infections and to define the degree of involvement as indicative of pathogenicity.

There is no uniformity of agreement among students of infectious disease, on the meaning of the term virulence. This is understandable if we consider that the concept has passed through several evolutionary stages. Pathologists, long before the etiological agents of disease were discovered, applied the term virulence in the sense of poisonous capacity without any implication of how the effect was produced. It would seem that virulence did not imply "poisonous-ness" as a laboratory worker would define it today, but rather it qualified the severity of the disease or epidemic. In the absence of knowledge relative to the intrinsic properties of infectious agents, definition of virulence had to be expressed as a particular end result in the host, usually death. With the isolation of several etiological agents and their cultivation apart from the host, bacteriologists soon recognized the components of virulence and distinguished between them by such terms as invasiveness, toxigenicity, etc. There was a greater tendency to stress the importance of factors unique to the organism with less emphasis on the characteristics of the host. To many, invasiveness became synonymous with virulence, not realizing that the property of invasiveness is an independent variable. In 1933, Topley (11) stressed the importance of using such terms as invasiveness to describe the various attributes on which virulence depends, and to "retain the term virulent in its correct sense of being poisonous, without any implication as to how the poisonous effect is produced, and to apply it to any organism which gives rise to rapidly fatal infection." During this period, Smith (12) in his classical essay on *Parasitism and Disease* directed attention to the importance of the relationship between the host and the parasite in determining the severity of disease. Although his was a broad ecological statement of the problem, it served to direct greater attention to the importance of host factors as essential attributes of the virulence phenomenon.

Ambiguities arising from restricted definitions of virulence have been pointed out recently (10). To the epidemiologist, virulence as a phenomenon in nature usually refers to the ability of a parasite which has reached the host to pass the superficial defenses, enter, multiply, and produce disease or death. The laboratory worker, on the other hand, thinks of a virulent parasite in terms of a small LD_{50}, under specified conditions. In reality, whether a parasite causes a lethal epidemic may depend on other properties which determine its ability to spread throughout a susceptible population. This quality might depend on its resistance to desiccation or its ability to be conveyed by a vector. A similar point has been stressed by Coburn (13). The virologist, on the other hand, has always thought of virulence in terms of an intimate host-parasite relationship [Burnet (14)]. The reason for this becomes obvious when we realize that all viruses appear to be intracellular obligate parasites and require a living cell in which to multiply. During the past decade there have been revolutionary advances in techniques which have enabled the virologist to isolate, purify, and better study the morphological and chemical characteristics of viruses. As a result, certain viruses have been found to have intrinsic properties which might be correlated with their ability to produce disease. The pioneering work of Burnet *et al.* (15) on the nature and mechanisms of bacteriophage-host relationships and the realization of their value in studying the fundamental problems of virus growth has stimulated workers, especially in this country, to employ these relatively simple systems for the study of mechanisms concerned with virus growth. No attempt will be made to review the literature in this field, other than to suggest the potentialities of such studies in the elucidation of the mechanism of virus virulence. Attention may be directed to the comprehensive pertinent reviews of Delbrück (16) and Cohen (17).

Virulence will be defined, for the purpose of this review, as a host-parasite relationship. In this sense, Dubos (1) has presented a practical definition of virulence as applied to bacteria, which we believe is equally applicable to viruses. This definition states:

> the ability of a microorganism to establish a pathological state in a given host is the summation of a number of different and independent attributes such as communicability, invasiveness, toxigenicity, etc. . . . Virulence is not a permanent intrinsic property of a given species. It expresses only the ability of a given strain of the infecting agent, in a certain growth phase, to produce a pathological state in a particular host, when introduced into that host under well defined conditions.

This is an ecological definition of virulence; it defines only in limited terms the attribute of the parasite and the host which together under well defined conditions determines the severity of disease. The present trend in the analysis of virulence is concerned with the elucidation of the mechanisms by which these various attributes influence the host-parasite relationship.

Infectivity and Communicability

Infectivity and communicability are not often considered as attributes of the virulence phenomenon. Laboratory workers usually implant the parasite within the tissues and, therefore, its ability to survive on the mucous membranes or to penetrate the epithelium is rarely taken into consideration. We are, however, interested in virulence as it applies to the parasite in nature and it seems important to discuss these properties of virulence.

Downs *et al.* (18) have made a comparative study of the susceptibility of several species of animals to *Pasteurella tularensis* by several routes of infection. Mice were highly susceptible to infection when the organisms were simply placed on an area of the shaved skin. The organism was also infectious by the conjunctival, intravaginal, and intranasal routes. Here then, we have an organism highly infectious by most of the natural routes. Unfortunately, there is no experimental evidence to explain the rapid penetration of the normal barriers by this parasite. With other organisms, however, the penetrative quality may be independent of the other attributes of virulence. Maaløe (19) has shown that a nonpathogenic strain of *Salmonella typhimurium* is capable of penetrating the intestinal wall and creating a primary focus with a facility equal to that observed for a pathogenic strain. The nonpathogenic strain, however, lacks the attributes of invasiveness or the ability to overcome the natural defenses of the host once it has become established. The converse can also be true as already pointed out [Coburn (13)].

Recently, there has been an increased interest in the development of techniques for the controlled exposure of animals in an attempt to measure respiratory pathogenicity. Rosebury and his colleagues (20) have published an excellent monograph which includes a description of cloud chambers and the results obtained in an extensive study on experimental air-borne infections. Of the

several bacterial agents tested, *P. tularensis* showed the highest degree of infectivity and lethality as tested in mice. An interesting point emerges, however, when one considers stability to atomization; *Brucella suis* and psittacosis virus (Borg) are definitely the most resistant and *P. tularensis* is the most labile. From an epidemiological point of view, resistance to desiccation is very important and when the stability-lethality and the stability-infectivity indices are calculated, *B. suis* has the highest respiratory infectivity and *P. tularensis* the lowest for mice. Elberg & Henderson (21) employing similar techniques exposed guinea pigs to aerosols of *B. suis* and *B. melitensis* and concluded that the respiratory route was little less effective than parenteral injection. *P. tularensis* (20) under these conditions has a very low communicability due to its susceptibility to atomization. When, however, a viable organism comes in contact with the tissues of the host it displays a high degree of infectivity. It must be emphasized that very little is known about the factors which stabilize these parasites in nature.

Such knowledge is not only of interest to the epidemiologist, but is equally important to those concerned with the preservation of living vaccines where the maintenance of at least a residuum of pathogenicity is important.

In addition, it is hazardous to extrapolate such results on respiratory pathogenicity to man. In this respect, however, there is suggestive evidence from laboratory outbreaks that psittacosis (22), tularemia (23), brucellosis (24), Q fever (25), and murine typhus (26) can infect man by the respiratory route. Recently, a definitive demonstration of an acute pneumonitis in a laboratory worker infected with psittacosis virus was described by Rosebury *et al.* (27). The incident and mode of infection were simulated employing *Serratia marcescens* and the results indicated that an aerosol of sufficient concentration of viable organisms reached the respiratory tract to account for the infection.

Young *et al.* (28) and Zelle *et al.* (29) have utilized controlled respiratory challenge to elucidate some of the factors involved in the infectivity for laboratory animals of spores of *Bacillus anthracis*. They presented additional evidence indicating that infectivity is an independent component of the virulence phenomenon. Four colonial variants of *B. anthracis* isolated from a single strain failed to show any difference in virulence when injected subcutaneously

into guinea pigs. When these variants were tested by the respiratory route, however, there was a marked difference in their virulence. It was concluded that the differences were due primarily to a factor which permitted the organisms to penetrate the tissues rather than their ability to establish a fatal infection after implantation had occurred. In addition, it was impossible to correlate colony morphology with infectivity, the two characteristics varying independently.

Lincoln *et al.* (30) have related this component of infectivity to the environment in which the bacteria are produced. It is possible to decrease the respiratory pathogenicity by producing the spores in corn steep liquor; this is immediately regained when the spores are grown in pepticase plasmolyzed yeast. These changes are probably due to temporary alterations in the expression of the genetic constitution of the culture caused by the environment; the changes occur in less than 11 to 18 cell generations and there is no detectable change in colony morphology. Subcutaneous tests in rats, with spores produced in corn steep liquor and of lowered respiratory virulence, showed no diminution in their ability to cause fatal infection. It is clear, therefore, that the infectivity component which permits the parasite to penetrate the normal barriers may be independent of the mechanisms whereby the organism invades and causes a fatal infection once it has entered the tissues. Nothing is known of the nature of these infectivity factors and further investigation is necessary.

Invasiveness

The property of invasiveness, as an attribute of virulence, directs attention to the various properties of the parasite which permit it to survive and multiply within the invaded host. Recent studies suggest that success or failure of the parasite in this respect is dependent to a large degree on the reactivity of the host.

Menkin (31), after a series of investigations on the mechanism of inflammation, put forth a new definition of bacterial invasiveness. In this concept, such substances as fibrinolysins and staphlocoagulase play no part in the process of invasion. The rapid walling off of a staphylococcal lesion is ascribed to the "intensity of local injury" brought about by the staphylococcal cell *per se* or its soluble necrotizing toxin. On the other hand, streptococcal infection

spreads and brings about rapid systemic reactions because the local reaction is less intense. Thus, the definition is quite paradoxical because the intensity of the local injury response and invasiveness vary inversely. Menkin has attempted to unify the concept of inflammation regardless of the nature of the irritant; this has necessitated rather broad generalizations and as a result Hadfield & Garrod (32) have criticized the concept because of the wide differences in the suppurative lesions brought about by the pyogenic cocci as contrasted to the acute inflammatory edema without leucocytic infiltration which occurs in anthrax infection. Adami (33) in 1909 recognized but one process of inflammation; no doubt Menkin (31) by his researches has contributed greatly to the understanding of the fundamental mechanisms, yet the manifestations of the inflammatory process are certainly various.

Studies on the pathogenesis of anthrax infection illustrate the importance of defining the host in respect to the type of inflammation and the degree of invasiveness [Cromartie *et al.* (34)]. Small quantities of edema fluid, extracted from anthrax lesions freed of the bacilli by filtration, when injected into the skin of normal rabbits produce an inflammatory edema characterized by a paucity of leucocytes (35). Chemical fractionation of the edema fluid resulted in the isolation of an inflammatory factor associated with or containing a large percentage of glutamyl polypeptide, which constitutes exclusively the capsule of *B. anthracis* (36, 37). This polypeptide neutralizes the antibacterial factors isolated from leucocytes suggesting a mechanism by which the parasite survives within the host (38, 39). The point to be emphasized is that an intense local injury is brought about by a toxic product of the parasite without setting in motion the fundamental stereopattern of inflammation as described by Menkin (31). These results illustrate the importance of taking into consideration the nature of the products of the parasite in accounting for the various manifestations of inflammation and their relationship to invasiveness.

Recently, Evans, Miles & Niven (40) have carefully investigated the mechanism of enhancement of invasiveness by epinephrine. There was an acute constriction of the arterioles which prevented the appearance of many of the characteristic features of the inflammatory response ordinarily induced by the presence of the parasite. In particular, there was a decrease in exudation and

diapedesis of leucocytes with restriction of the continuous supply of blood fluid. The absence of exudate precluded the influx of antibacterial substances and, therefore, the organisms continued to invade locally in the absence of the normal resistance factors of the host. Organisms isolated from animals after epinephrine-enhanced infection had not undergone any detectable change per se with respect to their ability to invade the normal host. The authors compared epinephrine enhancement with that produced by mucin; it would seem from the evidence as compiled and reviewed by Olitzki (41) that mucin acts by coating the bacteria and inhibiting their destruction by the natural defences of the host. Epinephrine differs from mucin in that it protects the parasite by inhibiting the mobilization of certain resistance factors. Evans *et al.* (40) discuss the possibility that dispersion not localization of the parasite is, under defined conditions, an effective defense. This paradoxical phenomenon was first described by Duran-Reynals and later discussed in his review on spreading factors in infection (42). It is possible by the concomitant intradermal injection of hyaluronidase, the enzyme responsible for depolymerization of the hyaluronic acid gel in the ground substance, to disperse the organisms over a wide area and thus to reduce the local critical concentration to a point where no lesion will develop and the animal will survive. Watson *et al.* (36) demonstrated the same phenomenon employing spores of *B. anthracis* together with the inflammatory factor described previously in this review. Sterne (43) has studied the effect of inflammation on the survival of guinea pigs infected with *B. anthracis.* The organisms were injected into early inflamed areas produced by such irritants as calcium chloride, saponin, or 20 per cent sodium chloride. All of the irritants tested increased the killing power of sublethal doses. Likewise, when attenuated bacilli were injected with 1 per cent saponin or 20 per cent sodium chloride a subimmunizing dose was raised to a full immunizing dose (44). Thus, we have an anomalous situation where inflammation and fixation enhance the multiplication and survival of these parasites.

The importance of inflammation to the activity of hyaluronidase in the skin has been convincingly presented by Hechter (45). The spreading effect of hyaluronidase using methemoglobin as an indicator was not only dependent on enzyme concentration, but

it was also related to the increase in the interstitial pressure produced by the injected fluid. Hyaluronidase administered into the skin in high concentrations but at a minimum volume and pressure actually failed to promote spreading (45). Hechter (45, 46) has logically pointed out that the ability of an organism to produce hyaluronidase *in situ* is not in itself sufficient to promote invasion within the skin. In view of the fact that hyaluronidase requires the presence of a local increase in the interstitial pressure to exert its maximum activity, the ability of the parasite to produce an inflammatory edema is of primary importance. Here, we have inflammation accompanied by lymphatic blockade and fixation, a host response normally utilized in preventing systemic invasion, being utilized by the parasite to augment its invasion throughout the skin. The frequent failure to correlate the *in vitro* production of hyaluronidase with invasiveness may reside in another quality of the organism associated with its ability to elaborate substances capable of producing inflammatory edema.

The complex enzymatic theory expounded by Haas (47) to explain invasiveness and natural resistance lacks confirmation. Hechter & Scully (48) demonstrated the *in vitro* activity of hyaluronidase inhibitor in serum but were unable to confirm the *in vivo* activity in skin. These results suggest that the mechanism described by Haas may not be important *in vivo*. Dorfman (49) has reviewed the literature on the action of serum inhibitors on hyaluronidase. Kinetic studies of the reaction between serum inhibitor and hyaluronidase revealed the process to be rapid and not linear with time. Also the rate of reaction was more rapid at lower temperatures which is not consistent with the idea that the inhibitor is an enzyme (50). The mechanism of serum inhibitor action on hyaluronidase remains a poorly understood phenomenon and nothing is known of the *in vivo* effect of this substance (49). These results indicate the ambiguities which may arise by extrapolating *in vitro* results to explain the *in vivo* mechanisms of invasion or natural resistance.

Maaløe (19) has carried out a series of investigations on invasiveness employing a unique experimental approach. Many of the attributes of virulence such as natural resistance of the host, infectivity, and toxigenicity have been maintained constant while studying invasiveness as the single variable. For these experiments

a pathogenic and a nonpathogenic strain of *S. typhimurium* derived from a single pathogenic strain were employed. There was no difference in their infectivity or toxicity as measured in the mouse; the difference in their pathogenicity was due to reduced invasiveness of the nonpathogenic strain. Analysis of the two strains showed no morphological or antigenic differences. There was a direct correlation between the invasiveness of the pathogenic strain and its resistance to complement; the nonpathogenic strain, on the other hand, was highly susceptible. Both strains were phagocytized to the same degree in the absence of complement. In contrast to the permanent mutation loss of pathogenicity, the pathogenic strain may show a temporary reversible loss of resistance to complement. Maaløe (51) produced maximum loss of resistance by cultivating the organisms in a high concentration of a carbon source and of ammonia as a nitrogen source. Although there was a close correlation between the invasiveness of the pathogenic strain and its resistance to complement *in vitro*, the question of *in vivo* activity of complement remained unanswered, especially in view of the reports claiming the insignificant activity of complement in the circulating blood. The early work of Fuchs on the similarity between coagulation and complement processes has led Maaløe (52) to re-examine the similarity between these processes in the light of recent knowledge. He cautiously points out that from several independent points of view there is a great resemblance between the coagulation and complement processes. Assuming this to be true, he has presented a working hypothesis which accounts for the latent antibacterial defence. The pathogenic and nonpathogenic strains of *S. typhimurium* are equally capable of establishing a primary focus within the host. Owing to mechanical or toxic cell destruction the processes of coagulation and complement formation are activited. This sets up a local zone of high antibacterial activity due to the formation of complement. The nonpathogenic strain being highly susceptible to complement is destroyed while the pathogenic strain, because of its high resistance, is capable of further multiplication and invasion.

Recently, Rammelkamp & Dingle (53) have reviewed the literature pertinent to the various intrinsic properties of the pathogenic streptococci, such as hyaluronic acid, hyaluronidase, fibrinolysin, and proteinase. It is becoming increasingly obvious that any at-

tempt to implicate any single factor as a central mechanism in the virulence phenomenon is over-simplification. Since Kass & Seastone (54) demonstrated the protection of mice against fatal doses of streptococci by the enzymatic destruction of the capsular hyaluronic acid, there has been a concerted effort to correlate the presence of the capsule with virulence (53). Although there is usually a close correlation, Pike (55) has shown that hyaluronic acid production does not assure high virulence. During the past year, Rothbard (56) has presented evidence which suggests that both the hyaluronic acid capsule and M substance contribute to virulence but that the M substance is of major importance. In relation to phagocytosis, encapsulated variants were slightly less susceptible than those from which the capsule had been removed with hyaluronidase. Encapsulated strains deficient in M substance were readily phagocytized while matt strains containing M substance were resistant to phagocytosis. Simultaneous injection of hyaluronidase with the streptococci followed by continued and prolonged enzyme therapy protected mice against 10 MLD. In contrast to this, a single injection of anti-M serum the day before challenge protected mice against 100,000 MLD. There was an additive protection when both enzyme and antiserum were combined. It seems quite clear from these studies that the capsule and the M substance protect the streptococci against the antibacterial forces of the host and thus contribute to the property of invasiveness. Indeed, Rothbard (56) points out there are probably unknown factors contributing to other attributes necessary for the development of virulence. For example, capsulated M-containing strains freshly isolated from patients and presumably virulent for man are invariably avirulent for mice. It is obvious, therefore, that mouse virulence depends upon an additional unknown factor.

Although considerable progress has been made toward the elucidation of the mechanisms by which certain parasites resist the defences of the host and manifest their invasive and other properties contributory to virulence, little is known in this respect for such organisms as *P. tularensis* and *Mycobacterium tuberculosis*. Downs *et al.* (57) have compared virulent and avirulent strains of *P. tularensis* without demonstrating any differences in their cellular components when analyzed electrophoretically and serologically. The virulent strain grows more rapidly on culture media

and in embryonated eggs; the attenuated strain, on the other hand, is more fastidious in its growth requirements and is less stable to dehydration. This difference, however, is an exception and not a constant characteristic of attenuated *P. tularensis.* Downs & Woodward (58) have studied several strains of low virulence and found them to grow well on artificial media. The determination of subtle differences between virulent and avirulent strains of these parasites should offer a profitable experimental approach leading not only to a better understanding of the various attributes of virulence but to the elucidation of the nature of immunogenesis.

The early description of methods for the dissociation of the tubercle bacillus by Petroff & Steenken (59) stimulated many investigators to a similar attack on the problem of pathogenesis. Recently, many of the experimental difficulties, which in the past prevented definitive results, have been resolved. The development of culture media by Dubos and his colleagues (60, 61), for more rapid and uniform growth, has made it possible to prepare challenge doses with a constant number of viable cells; the recent studies on the mouse as an experimental animal (62) have permitted a more thorough correlation of virulence with certain morphological characteristics. Applying these techniques, Middlebrook, Dubos & Pierce (63) have demonstrated the ability of cultures of mammalian tubercle bacilli, when cultured under specified conditions, to form parallel cords; virulent strains invariably showed these morphological characteristics. On the other hand, avirulent strains cultivated under identical conditions grow in a much less oriented fashion. The authors (63) have postulated that these differences may reflect the presence of a substance which causes the bacilli to adhere to each other and unearths the possibility that this material is responsible for some attribute of virulence. The cytochemical test developed by Dubos & Middlebrook (64) is designed to differentiate between these morphological types. The principle of the method involves the ability of the virulent cells, or those which show cord formation, to bind the dye, neutral red, in the form of an anion under specified conditions. Although the cells are suspended in an alkaline buffer and the unbound dye is yellow, the dye taken up by the cells is purple red; those strains which fail to show cord formation do not fix the dye. It is interesting to speculate concerning the similarity of this phenomenon to that described by Sabin & Feldman (65) for toxoplasma. When normal extracel-

lular toxoplasma are stained with methylene blue in a buffer solution at pH 11, the cytoplasm takes up the dye; the addition of an immune antibody plus a heat labile factor present in normal serum modifies the cytoplasm in such a manner that it fails to stain. The authors have applied the technique to the measurement of these antibodies in sera from recovered patients and suggest the possible application to the detection of bactericidal and viricidal antibodies. Antibodies capable of blocking the reaction described by Dubos & Middlebrook (64) have not been described and this interesting mechanism remains obscure. If, however, the factor responsible for binding the neutral red within the cytoplasm constitutes some component of virulence, it seems rational to postulate that animals, with acquired resistance, possess an antibody capable of neutralizing this mechanism.

On the basis of somewhat similar reasoning, Raffel (66) has designed experiments for extracting specific cellular antigens responsible for some portion of the virulence phenomenon. The isolation from the tubercle bacillus of a toxic carbohydrate-lipid fraction by paraffin oil extraction [Choucroun (67)] and its possible relationship to the Boivin type antigen led Raffel (66) to investigate these substances. These complexes isolated from the H37 RV strain, by trichloracetic acid and urea extraction, were found to augment generalized infection when injected into guinea pigs concomitantly with small numbers of tubercle bacilli. It seems logical, therefore, that these antigens play some role in the invasiveness of this parasite. The absence of such antigens in attenuated tubercle bacilli would strengthen this contention; their presence, however, would not negate their importance as contributing factors to the virulence of the tubercle bacillus. As already pointed out, it is rare indeed when a single factor accounts for all the components of virulence.

Toxigenicity

In the preceding sections, we have discussed the various components of the virulence phenomenon which deal with the ability of the parasite to establish itself within the host and multiply. As pointed out by Topley (11) the mere presence of the parasite within the tissues or blood of the host cannot explain its harmful effect.

Bacteria.—For those infections characterized by bacteremia,

such as anthrax, death has been often attributed to mechanical blocking of capillaries. Such an explanation is not compatible with the results obtained by Bloom *et al.* (68) showing that rabbits may die from the infection with relatively few organisms in the blood. Most investigators agree that the manifestation of virulence in many infections can be explained by the attribute of toxigenicity (1, 4, 11); this component of virulence determines the degree to which the metabolic products of the parasite or its soluble products interfere with the normal functions of the host. The expression may be a pathological lesion or death.

Toxigenicity, like infectivity or invasiveness, is not necessarily an independent character of virulence. For instance, damage to the epithelial wall of the intestinal canal brought about by toxic products of the parasite may facilitate infectivity (19). We have in such cases a close correlation between infectivity and toxicity. In line with Menkin's reasoning (31), the converse may be true. In other words, the toxicity of the parasite may incite inflammation in the host at the point of entry and impair or prevent invasion. One might conjecture that the rapid infectivity and invasiveness of *P. tularensis* is an expression of the innocuousness or lack of toxicity to the tissues and that toxigenicity only becomes evident after the parasite reaches and proliferates in specific tissues of the host. Toxigenicity may also be independent of the other attributes of virulence. In this respect, variants of *S. typhimurium* may have the same toxigenicity but differ widely in their invasiveness (19). Although the role of the toxin of the diphtheria bacillus is well recognized in the pathogenesis of the disease, the reason for the greater pathogenicity of the gravis strains is still lacking. Hewitt (69) has studied the toxigenicity and other properties of different serological types. There was a close correlation between lethality and the ability of the cultures to produce skin-reacting toxin in broth cultures. The killing power in guinea pigs apparently is due entirely to the attribute of toxigenicity. The author points out the desirability, however, of reserving the term virulence to describe the bacteria capable of multiplying from very small inocula; large inocula are necessary to produce early lethality in guinea pigs. Virulence in this sense would be represented by other components, such as infectivity and invasiveness, as well as toxigenicity. In agreement with previous investigations, the toxigenicity

of the gravis strains was not greater than that of the mitis strains and there was no explanation for the greater clinical severity of disease produced by the gravis strains. In Type III, the virulent strains were hemolytic while the avirulent strains were not. Since virulent gravis Type I strains were both hemolytic and non-hemolytic this correlation does not hold. Differences in the antigenic specificity of the toxins do not explain the variation in the clinical severity of the disease; a single antitoxin protected guinea pigs against the fatal effects of living cultures of all the serological types tested. That another attribute such as invasiveness rather than toxigenicity might explain the discrepancies between the virulence of these strains might be suggested by the work of Frobisher & Parsons (70). Rabbits injected with broth cultures of living, nontoxigenic strains of *Corynebacterium diphtheriae* develop significant resistance to subsequent injections of living cultures in the absence of detectable antitoxin in the blood. Recently, Frobisher & Updyke (71) have extended these observations. The absence of antitoxin in the blood suggests that the acquired protective mechanism of the host is directed at some invasive quality of the parasite, exclusive of toxigenicity. Further work is necessary to elucidate this mechanism.

Current investigations are confirming the original suggestion of Topley (11) that an understanding of the mechanisms of toxigenicity will be resolved by a chemical approach. The chemistry of bacterial toxins is reviewed in this issue of the *Annual Review of Microbiology* and, therefore, the subject will be dealt with briefly.

The interesting hypothesis of Pappenheimer & Hendee (72) on the possibility that cytochrome-*b* of the diphtheria bacillus is composed of iron, porphyrin, and toxin and the assumption that the toxin interferes with the functioning of cytochrome-*b* in the tissues of the host is a new and valuable approach to an understanding of the mechanisms of toxigenicity. Enzyme activity as a mechanism of toxigenicity was first described by MacFarlane & Knight (73) when the alpha toxin of *Clostridium welchii* was discovered to be a lecithinase specifically catalyzing the hydrolysis of lecithin. It would seem that this toxin combines the necrotic, hemolytic, and toxic properties all in one molecule. Miles & Miles (74), studying the lecithinase of *Clostridium bifermentans*, have described preparations which are hemolytic but only slightly toxic.

All attempts to neutralize the lecithinase of *C. welchii* without interfering with the other properties, employing antilecithinase from *C. bifermentans*, were unsuccessful. Oakley *et al.* (75), MacFarlane (76) and Mason (77) have extensively studied the toxins from these and other clostridia. The importance of collagenase, an enzyme capable of destroying connective tissue and associated with the lethal toxins in the culture filtrates from many clostridia, has been discussed by Evans (78). The lethal toxin of *Clostridium histolyticum* and collagenase activity are not the same. It would appear, therefore, that the enzyme contributes to invasiveness, especially since anticoagulase will inhibit the activity of the culture filtrates.

Bernheimer (79) reviewed the literature on what he terms the rapidly or acutely acting toxins. Specifically, he has discussed the two hemolytic toxins, streptolysin O and streptolysin S produced by *S. pyogenes* and first distinguished by Todd (80). Kinetic studies on the hemolysis of erythrocytes by these toxins suggest that they function catalytically in a manner comparable to enzymes. Injected into mice, streptolysin O induces a fatal toxemia; the mice do not die of an intravascular hemolysis and it is obvious that the toxins act on cells other than those of the blood. The interesting observations of Bernheimer & Cantoni (81) on the cardiotoxic action of these substances in the isolated heart of the frog and the more recent evidence (82) of a similar mechanism in the mammalian heart represents a new departure in the elucidation of the mechanism of toxigenicity.

From these few examples, it is becoming obvious that enzymes and enzyme inhibitors of the parasite may explain many of the manifestations of toxicity.

Rickettsiae and viruses.—The past decade has brought initial evidence of toxic substances intimately associated with viral and rickettsial parasites. Gildemeister & Haagen (83) showed that heavy suspensions of murine typhus rickettsiae injected parenterally into mice caused a fatal issue within a few hours. Similar properties demonstrated with the rickettsiae of scrub, as well as epidemic typhus (84 to 87), have a counterpart among viruses of the lymphogranuloma-psittacosis group (88), influenza (89 to 92), and Newcastle disease (93, 94, 95). Among the characteristics of these intoxications in mice are early onset, sometimes after 1 to

2 hr., with signs of weakness, dyspnea, prostration, convulsions, and death in 18 hr. or less after injection in the case of the rickettsiae. Toxic expressions and deaths from viral agents generally develop only after a day or two. Animals which survive the toxic illness may recover and remain well for a few days only to develop infection and die. The toxins or toxin-like substances, although extremely labile, have not as yet been found to be dissociable from the respective infective agents. Like various bacterial toxins, the viral and rickettsial poisons when inactivated or detoxified by formaldehyde elicit highly specific protective antitoxins.

While the symptoms engendered in experimental animals resemble the toxic clinical manifestations sometimes seen in the natural diseases (e.g., influenza, Newcastle disease), laboratory studies have not elucidated the mechanisms of the intoxications nor of their roles in virulence in the several rickettsial and viral diseases. However, Clarke & Fox (96) present evidence that the toxicity of rickettsia-infected yolk sac material for mice is dependent upon a factor which gravely alters vascular permeability and that death results from shock.

Separable from certain of the viral and rickettsial agents are a number of so-called soluble antigens, serologically specific but lacking the infectivity and the immunogenic dimensions of the parasitic entity. Working with vaccinia, Craigie (97) demonstrated the first such soluble antigen, now designated LS. Another antigen, NP, extracted from vaccinia, has properties duplicated by somewhat similarly derived substances from other of the larger viruses and from various rickettsiae. These materials are neither demonstrably noxious to the host nor are the antibodies incited by them protective against the respective intact agents.

Nevertheless, advances in knowledge continue to reveal parallelisms between the properties and behavior of the various products and components of the viruses and rickettsiae and of the bacteria. Some of these features and characteristics may obviously bear upon or influence virulence. Hereditary transmission to noncapsulated pneumococci of the capacity for heterologous capsule formation (98) appears to have been simulated in the case of certain rabbit tumor viruses. Berry & Dedrick (99) reported that rabbits treated with a mixture of active fibroma virus and heat-inactivated myxoma virus developed, not fibromata, but

typical myxomatosis. Although the long recognized and much studied property of bacterial hemolysis would appear to play a minor or negligible role in the genesis and production of disease with the exception of certain gas gangrene infections, yet hemolytic activity *in vitro* by mumps virus has also recently been demonstrated (100). This enzyme or enzyme-like property behaved similarly in several respects to the factor which promotes erythrocyte agglutination, and which is identical in a number of viruses, e.g., influenza (101, 102) and Newcastle disease (103). Associated with the process of sensitization to and/or hemagglutination there occur more or less profound changes of the surface of the erythrocyte, presumably as the result of interaction of the virus-enzyme and the cell receptor substance. This alteration is so marked as to result in a change in antigenic character of the erythrocyte (104, 105) and has been postulated as indicating a pattern of alteration incidental to or even requisite for virus invasion of other specifically susceptible cells (106). In this direction, Wang (107) observed that the capacity of a line of influenza virus to agglutinate chicken erythrocytes might be a better indication of its pathogenicity for mice than would its capacity to infect embryonating eggs. The mouse pathogenic virus had a much higher growth rate in the mouse than a nonpathogenic line and it was suggested that this might explain the more ready adaptation of some virus variants to another host species. One might ask whether this phenomenon may indicate a mode or mechanism possibly of more than minor significance, modifying the virulence of viruses, especially those possessing pneumotropism. That resistance to heat or other adverse environmental factors may constitute an undefined but quite obvious characteristic related to virulence of *P. tularensis* has already been cited (18). Armstrong (108) had correlated the property of virulence of vaccinia for rabbits with resistance of the virus to relatively high temperatures. More recently the greater resistance to loss of viability as well as to inactivation of hemagglutinative function among numerous strains of Newcastle disease virus has shown a direct relationship and also an overall correlation with virulence for chickens and with the time and locality of isolation of the virus strain (109).

Biological Synergism

The combined activity of virus and bacterial infection often

results in an aggravated form of disease. It has long been recognized that "virulent" smallpox infection may owe its severity to complication with bacterial infection. Recently, Lack (110) concluded from his study of dual infection with staphylococci and vaccinia that the latter agent is spread in proportion to the quantity of hyaluronidase produced by the concomitant staphylococcal infection; hyaluronidase-producing staphylococci, which induce minimal lesions when injected alone, are spread in proportion to their hyaluronidase production when introduced with vaccinia virus. The suggestion is quite generally accepted that the 1918 pandemic of influenza differed from less communicable and generally milder epidemics of the disease by the greater tendency of the "pandemic" virus toward association with hemophiles and pathogenic cocci. Shope's classical studies of the swine influenza syndrome (111) have established a sound interpretation of virus-bacterial synergism in determining the ultimate clinical picture. Likewise, the role and mechanism of bacterial or other provocative stimuli in activating such a latent virus infection were well shown.

That communicability, as a component of virulence, rather than virus invasiveness or colonization is favored at times by virus-bacterial synergism is suggested by certain circumstances in some maladies. Obviously, if the virus requires a "robust" host cell substrate prior disturbances of cellular metabolism could adversely affect infection by the virus. Prior or secondary microbial invasion or activity may, however, magnify the disturbance of health in various aspects and, in so doing, favorably influence the dissemination of the virus. In diseases such as influenza, secondary bacterial involvement usually increases the quantity of respiratory exudate, with resultant better protective coating of the virus. This factor, coupled with accelerated sneezing and coughing, facilitates communication of the infection to fresh hosts. Definition of the virus interference phenomenon among yellow fever virus strains of different tropisms (112) stressed that this mechanism may greatly mediate virulence among more or less closely related viruses. In fact, the blocking effect of one virus species upon a relative may approach in efficiency the interference engendered by a particle of the homologous virus. Nevertheless, evidence of virus synergism in the simultaneous infection of the same cell has been demonstrated with viruses of dissimilar properties as, for example, the case of two agents with apparent differential predilections for

the nucleus and the cytoplasm, respectively, as indicated by the position of the virus inclusions which are produced (113, 114, 115). More recently Ginsberg & Horsfall (116) suggest that the available information on the interference phenomenon with viruses supports the possibility that the intracellular metabolic system of the host, not merely the cell surface receptors, is the major factor upon which the phenomenon depends. They propose, furthermore, that the metabolic requirements may not be identical even for viruses which multiply in the same host cells.

In vivo Environment and Virulence

In the preceding sections virulence has been discussed in terms of the host-parasite relationship. In this respect, the reaction of the host to injury initiates many physiological processes and the parasite must be capable of survival in this dynamic environment. Simultaneously, the parasite is capable of undergoing variation. The fundamental mechanisms involved in this situation have been clarified by the bacterial geneticists and several excellent reviews have recently appeared (117, 118, 119). There is sufficient evidence to indicate that the increase of virulence of the parasite by serial passage in the host is dependent upon selection of spontaneous variants (mutants); these variants are capable of proliferation in an *in vivo* environment because of certain inherent properties already discussed. Braun (118) has summarized his findings which bear upon elucidating such a mechanism for *Brucella abortus*. Under standard environmental conditions this organism undergoes spontaneous smooth (S), intermediate (I), and rough (R) variation which can be measured and expressed in terms of dissociation indices. Normal sera from susceptible species such as the rabbit, guinea pig, cow, and man contain a heat stable, filterable factor associated with gamma globulin which selectively suppresses the establishment of the nonsmooth types. On the other hand, sera from the more resistant mouse, rat, and various species of fowl lack the selective factor and, in the presence of these sera, there is a rapid dissociation to the nonsmooth avirulent forms. It is important to note that this globulin fraction is not associated with complement or other bactericidal properties of serum. Braun & Hauge (120) have shown that these selective factors express themselves *in vivo* as well as *in vitro*. Only S variants have been isolated

from the relatively susceptible guinea pig and rabbit, whereas all variant forms have been recovered from the spleen of the more resistant mouse. These results support the suggestion that susceptibility is correlated with the presence of a globulin serum factor suppressing the establishment of nonsmooth, usually avirulent types and the absence of this factor is correlated with increased resistance (118). Hoerlein (121) has cultivated *Staphylococcus aureus* in the presence of gamma globulin fractions and reports comparable results to those obtained with *B. abortus*. Further investigations may reveal this phenomenon to be operative in other host-parasite relationships.

B. anthracis cultivated on ordinary culture media is rough and nonencapsulated. When injected into susceptible animals, the bacillus undergoes morphological changes associated with capsule formation and, in addition, one may extract a protective antigen from the local lesions (122). That these substances constitute a specific function is only a suggestion, but by analogy with other organisms one may postulate an important role in some aspect of virulence. Organisms from the local lesions cultivated on ordinary media immediately revert to the rough forms. It becomes obvious, therefore, that any attempt to isolate virulence factors from cultures in the usual media will fail. In other words, the organisms growing in ordinary culture media are only potentially virulent; the production of substances necessary for the manifestation of virulence takes place only in the tissue environment. Gladstone (123) has been able to simulate these environmental conditions *in vitro* by cultivating the organisms in normal plasma under well defined conditions. These experiments led to the production of the protective antigen *in vitro*. Recently, Heckly & Goldwasser (124) have extended these observations and successfully produced the protective antigen in a simplified medium consisting of denatured sheep serum albumin and ox serum ultrafiltrate. The striking morphological changes are comparable to those observed in the skin lesions of rabbits (34). No experimental evidence has been presented to explain the mechanism of this rapid transformation. It is tempting to speculate, however, on the possibility that processes similar to adaptive enzyme formation (125, 126) may play an important part in this phenomenon. The adaptive formation of hyaluronidase by certain streptococci in the presence of the sub-

strate, hyaluronic acid (127), sets a precedent for such speculation. Block (128) cultured tubercle bacilli in chicken embryo extract and quantitatively increased the cord formation which was associeted with the virulent bacilli (63). Avirulent strains growing on ordinary media form nonoriented clumps but when cultivated in the presence of chicken embryo extract the well defined cords considered characteristic of the virulent types were observed. In the absence of the extract, there was complete reversion to the avirulent type.

These few illustrations suggest that the *in vivo* environment as well as specific factors isolated from the normal host may play an important role in determining the qualities which endow the parasite with one or more aspects of virulence.

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TYPES OF ACQUIRED IMMUNITY AGAINST INFECTIOUS DISEASE[1]

By Sidney Raffel

Department of Bacteriology and Experimental Pathology, School of Medicine Stanford University, Stanford, California

In this review attention will be centered upon the fundamental immunological considerations which determine protective immunization against infectious organisms; that is, the modifications in the host which serve as protective devices and the constituents of the infectious agents responsible for the induction of resistance. Only the essence of clinical statistics will be dealt with; sufficient to indicate the practical usefulness of the individual immunizing procedures.

On the basis of several thoroughly worked-over examples, we are inclined to generalize acquired resistance as being the result of a specific antibody response to an antigen possessed by the infectious agent. Clear evidence for this mechanism is actually limited to relatively few well-known examples—the pneumococcus, the hemolytic streptococcus, the gram negative enteric bacilli, the antigenic toxins, and perhaps some of the viruses, such as those of influenza, rubeola, and mumps where, when the virus is in the blood phase at least, antibodies may effectively inhibit the disease. In many of the bacterial diseases it has not been possible as yet to correlate the presence of antibodies with the immune state, and it seems obvious that with those agents—the viruses and rickettsiae—in which the relation of organism to host is intracellular, antibodies may never have sufficient opportunity to act even though they might be able to influence the agent if the chance were afforded.

In the case of the bacteria mentioned above, the immunizing factors appear also to be important "virulence factors." Antibody immunity therefore serves to overcome or neutralize an aggressive property of the infecting organism. Although this kind of joust seems entirely reasonable from analogies in human activities, it is certainly conceivable that the host may "choose" to ignore the

[1] This review covers the period from approximately January, 1945 to March, 1949.

aggressive properties of the organism and instead to direct its acquired defense against some vital function of the agent; for example, a respiratory enzyme system. Or it may simply modify itself as a medium, failing to support the multiplication of the agent, as may be the case with certain intracellular parasites. These thoughts are suggested by the fact that avirulent forms of some bacteria and viruses (i.e., without "virulence factors") are suitable immunizing agents, inducing protection against the virulent forms even better than the killed virulent agents themselves. Other explanations for these observations are available; for example, in the case of the bacteria, that the process of killing the virulent organisms for vaccine preparation introduces chemical changes in important antigens which makes them unsuitable as immunizing agents, or that, in the case of the viruses, the concentration of killed viral antigen is insufficient as an immunologic stimulus, the living avirulent organism multiplying to supply enough of this. These factors probably make themselves felt in certain instances, but to the reviewer it seems desirable to remain receptive also to evidence for the concept that less familiar mechanisms than the conventional antigen-antibody relationship may enter into acquired resistance in some diseases. A limited number of bacterial and toxic diseases of man and animals will be considered in this review. The selection is random, determined by alphabetical order in a complete list which is being reviewed from the same standpoint. Even this casualness of choice, however, gives evidence of elements concerned in resistance which do not readily fit the conventional picture.

In some instances, adjuvants are employed in practical vaccination; Freund (1) has recently reviewed certain of these.

Anthrax

The protection-inducing factor.—Pasteur in 1881 (2) first demonstrated the immunizing potentiality of living anthrax bacilli attenuated by cultivation at raised temperatures. In 1904 Bail (3) showed that the bacteria-free edema fluid from anthrax lesions could actively immunize animals. This immunizing factor appears as the result of the activity of avirulent as well as virulent organisms in the tissues, but it is not found in filtrates of ordinary anthrax cultures. As opposed to the evident immunizing properties of living avirulent bacilli and of lesion fluid, there is general

agreement that vaccines prepared from killed virulent bacteria are not satisfactory immunizing agents, although they provoke antibody responses against certain of the well-defined antigens of the cell. These fundamental points have been amply confirmed during the intervening years. Much of the recent work has been carried out by Grabar & Staub (4 to 9) in France, by Gladstone (10, 21) in England and by Cromartie and his associates (11 to 17) in the United States.

The immunizing factor present in edema fluid has been characterized by Grabar & Staub (8) as a substance containing a polysaccharide. This they believe to be the "a" polysaccharide (one of 2 present as somatic carbohydrates in the bacterial body), apparently in association with a protein. The polysaccharide itself was found to be inactive as an immunizing agent and was considered to be a hapten. Cromartie *et al.* (12, 13) have encountered difficulty in attempting to fractionate the lesion extracts with ammonium sulphate; the protective antigen appeared in all fractions. By cold ethanol precipitation it was possible to concentrate the antigen chiefly in the fraction precipitated by 25 per cent alcohol, and the remainder at concentrations between 25 per cent and 40 per cent. Electrophoretically, the immunizing fraction occurred in the α-, β-, and γ-globulin components of the extract; the albumin component was devoid of activity. The antigen was heat labile (57°C. for 30 min.) and destroyed by trypsin.

It is well known that ordinary culture filtrates of *B. anthracis*, unlike the saline extracts of lesions, do not contain an immunizing substance. Schilling (18) and White (19) have reported some success in immunization employing cultures containing body fluids. Recently Gladstone (10) has described the elaboration of the protection-inducing factor by *B. anthracis* in medium containing plasma. This occurs independently of the virulence, capsulation or sporulation of the organism employed. The immunizing agent was present in filtrates of cultures only during a limited period of growth, little of the immunizing factor being present at 12 hr.; at about 18 hr. it occurred in highest concentration, and had disappeared by 48 hr. Its disappearance depends not upon its lability, but upon the activity of a proteolytic enzyme released from autolyzed bacteria. Like the edema fluid factor this immunizing agent is also heat labile, and it too induced optimum protection in rabbits and sheep. This immunizing factor did not occur as a constit-

uent of the organism itself even at the most favorable time for its demonstration in the medium. The constituents of the medium responsible for the production of the factor were a nondialyzable component, probably albumin [confirmed by Heckly & Goldwasser (20)], and a dialyzable substance which could be replaced by sodium bicarbonate. In a later paper (21) Gladstone has described a method for obtaining greater yields of immunizing factor by preventing autolysis of bacilli and consequent liberation of proteolytic enzyme. This is accomplished by carrying out cultivation in cellophane sacs, with renewal of medium constituents by dialysis together with provision for complete oxygenation.

Gladstone (10) considers that albumin may be the protein portion of the polysaccharide-protein antigen which Grabar & Staub (8) have described. Note, however, that Cromartie *et al.* (13) found that the albumin moiety in lesion extract did not carry the immunizing factor, but rather that this migrated with the globulins in the electrophoresis cell. A recent reappraisal by Heckly & Goldwasser (20) indicates that a heat stable substance associated with electrophoretically purified albumin serves in culture for the production of the immunizing factor, and they suggest that when isolated the latter factor will prove to have an electrophoretic mobility of its own.

Now if we provisionally accept from these data the concept that a polysaccharide of the bacillus (avirulent or virulent) may, under conditions of growth established by living tissues or in culture medium containing plasma, combine with a substance associated with albumin to form a protective antigen, we have the conventional picture of antigen-antibody relationships familiar from other examples; but certain difficulties arise to complicate this concept. The first is the fact that Gladstone's (10) culture factor may induce solid resistance in the rabbit with no indication whatever of an antibody, either in the blood or in extracts of spleen and other tissues, capable of passively transferring protection or of reacting in *in vitro* tests. The same may be said of lesion extract immunization. Apparently a protective serum factor appears only after hyperimmunization (10, 13, 21). To go a step farther, the serum of animals so immunized contains antibody which precipitates the somatic polysaccharides of the bacillus, but this antibody has no relationship to protective resistance. It was shown by Grabar & Staub (4) that while the precipitating antibodies of

antianthrax horse serum occur in the euglobulin portion of the serum, the protective factor is limited to the pseudoglobulin. Furthermore the protective antibody can not be absorbed with either the bacillary polysaccharides or the capsular antigen (5). In fact, the protective portion of the serum exhibits no *in vitro* reaction with any component of tissue extract, plasma culture factor or the bacillus itself, nor is it possible to absorb or neutralize the serum factor with any of these substances, including living encapsulated bacilli (10, 13, 22). The paradoxical finding by Grabar & Staub (8) that the immunizing factor of edema fluid could be precipitated by a nonprotective antibody (antipolysaccharide) of horse serum was explained by these investigators on the basis that the entire antigen necessary for immunization (polysaccharide-protein) is flocculated by the antibody directed against the polysaccharide portion, while the protective "antibody" (presumably antiprotein) itself is incapable of precipitating or otherwise reacting with the antigen.

In view of the facts that an antigenic status for the immunizing factor has not been clearly established, that resistance may be invoked without concomitant humoral immunity, that when methods are employed to create humoral (transferable) immunity this is not associated with an antibody which can be demonstrated by any *in vitro* test or absorption procedure, and that those serologically manifest antibodies which are produced bear no relationship to resistance, it seems evident that more work will be necessary before the relationships in resistance to anthrax can be safely envisaged within the conventional antigen-antibody concept.

Relation between the protection-inducing factor and virulence of the anthrax bacillus.—Bail & Weil (23) in 1911 described the presence in anthrax lesion extracts of an "aggressin," a substance aiding the establishment of infection by neutralizing the native defense processes of the host. No indication of a forthright damaging effect upon tissues was noted. Other early workers (24, 25) related this effect, as well as the occurrence of edema, to the capsular substance of the virulent bacillus. Tomcsik & Ivanovics (27a) and Ivanovics with Erdös (26) and with Bruckner (27) some years later characterized the capsular material of the bacillus as a polypeptide composed of about 50 D(-) glutamic acid units—one of the exceptions to the more frequent polysaccharide structure of capsular antigens. Hanby & Rydon (28) more recently have reported this

molecule to be made up of alpha peptide chains of 50 to 100 D(-) glutamic acid residues joined together by gamma peptide chains of D(-) glutamic acid residues. As indicated before it has been a unanimous finding that this capsular material is not immunologically related to the protective factor in antianthrax serum, and the antibodies in such serum directed against the capsular material have no part in protection.

Watson *et al.* (13, 16) have lately isolated a material from anthrax lesion extracts which is separable from the immunizing factor by adsorption with colloidal calcium phosphate, and by its electrophoretic mobility. This highly mobile component induces local pathological changes following intracutaneous injection very similar to those resulting from the injection of anthrax spores, including edema, fragmentation of collagen, hemorrhage, and infiltration by small numbers of polymorphonuclear and mononuclear cells. This substance also inhibits the activity of an anthracidal factor present in the tissues of normal animals (14). This twofold role in pathogenesis indicates its probable importance as a "virulence factor." Interestingly enough, analysis showed this lesion-producing substance to be chemically related to capsular polypeptide. In electrophoretic analysis the two substances showed similar mobility curves at various pH levels. Compared with the capsular polypeptide on the basis of biological activities, some differences were noted: the capsular material failed to induce tissue damage and failed to inhibit blood clotting, while in both these respects the lesion factor was active. Both substances, however, inhibited the anthracidal activity of normal tissue extracts equally well. They may thus be assumed to be the equivalent of Bail's "aggressin."

In summary then, a bacillary capsular substance, which is present also in anthrax lesion extracts in somewhat altered chemical form, possesses the properties of neutralizing the anthracidal factor extractable from the tissues of normal animals, and of causing tissue changes similar to those induced by the organisms themselves. This substance is antigenic; precipitating antibodies against it appear in immunized animals. Yet this substance is entirely distinct from the lesion (and plasma culture) factor responsible for the induction of acquired resistance.

The mechanism of acquired resistance to anthrax.—The mechanisms through which acquired resistance to anthrax function are

not entirely clear. Cromartie *et al.* (11, 12) have made histological observations of cutaneous areas injected with spores in normal and immunized rabbits and other species of animals. In the normal rabbit (11) developing lesions revealed germination of injected spores and proliferation of bacilli during the first 4 hr. During this interval there evolved the same damage and inflammatory response as described above in relation to the tissue damaging factor extractable from lesions. Multiplication of bacteria continued until the infection was terminated by death, and practically all bacilli remained well capsulated throughout this process. Only a few leucocytes ventured into the damaged areas.

In immunized rabbits (12) firm cutaneous nodules developed. Microscopically, there was noted a gradual increase in numbers of bacilli at 4 to 8 hr. after injection, and these were capsulated organisms. At 12 hr. even more bacilli were seen, but now the capsules were lacking and the bacillary bodies stained unevenly. Although large numbers of leucocytes collected about the foci of bacteria, phagocytosis was very occasional and of no significance to the obvious destruction of bacilli which was now in progress.

This sequential picture of the functioning of acquired resistance is the same as that seen in normally insusceptible species of animals such as the dog and swine (11). Cromartie and his co-workers (12) therefore suggest that the fundamental process of resistance in both instances is the same, in essence an interference with the formation of the bacterial capsule. This suggestion stems from the facts that even though anticapsular antibody per se does not damage bacilli, yet the first stage in their eventual destruction is the disappearance of their capsules. Presumably then, since this sequence is observed in normal insusceptible as well as in immunized susceptible animals, the substance which effects interference with capsule formation and death of the bacterium is the anthracidal factor of normal tissues which has been isolated and characterized by Bloom *et al.* (14) as a basic polypeptide with high lysine content. This substance in isolated form was shown to be active in the destruction of anthrax organisms *in vitro*, and to act as a chemotherapeutic agent for anthrax *in vivo*. [It acts also upon other bacteria (15).] The substance is counteracted by the capsular antigen of the bacillus (i.e., Bail's "aggressin" or the virulence factor). In animals with native resistance the concentration of the anthracidal factor is apparently sufficient to be effective

despite the neutralizing tendency of the capsular substance, whereas susceptible animals, which also possess anthracidal factor, possibly do not mobilize it in sufficient quantity to be effective (although some of the bacilli in the susceptible animal do decapsulate and deteriorate).

The function of the anthracidal factor in preventing capsule formation seems probable on the basis of the evidence available. Whether this effect leads to the subsequent death of the cell is speculative.

Now, in what specific manner does acquired resistance against anthrax actually serve the body? The simplest concept would be that antibody to the capsular antigen neutralizes its ability to destroy the anthracidal polypeptide, but this antibody has no such effect, as has been discussed. The humoral protective factor (antibody?) is induced by another immunizing substance (antigen?) and does something which permits extracellular destruction of bacilli to occur in an ordinarily susceptible species of animal. It seems obvious that the resistance factor does not interfere with the proliferation of the organisms, since these multiply progressively during the first 12 hr. after infection. Nor does it appear to localize organisms until such time as sufficient anthracidal factor may accumulate from disintegrating leucocytes (which gather in large numbers and undergo necrosis on a large scale) to effect its activity, for there is no obvious agglutination of bacilli to implement such a view, and furthermore Bloom *et al.* (14) have determined that bacteremia was present in immunized animals as late as 72 hr. after infection. No hint is available concerning the role in the process described of the acquired resistance resulting from vaccination.

Résumé of relationship of protection-inducing substance, virulence factor and acquired resistance in anthrax.—So far as a summary can be made on the basis of available information, the relationships between immunizing factor, virulence factor, and the mechanism of the acquired immune state may be tentatively viewed as follows: In the normal, susceptible animal a potential defense mechanism (an anthracidal basic polypeptide) is effectively neutralized by the capsular material of the bacillus ("virulence factor," a polypeptide composed of glutamic acid). The organisms then proliferate and produce progressive disease. Acquired resistance, however, may be induced by another isolatable extra-

cellular factor (antigen?) produced by multiplying bacteria in tissues or in plasma-containing medium; this stimulates in the host a response (antibody?) which in some manner may permit a more effective mobilization of anthracidal factor. The latter interferes with capsule formation (it does not attack the capsule per se) and kills the cell. Thus, the "virulence factor" of the bacillus neutralizes a normal defense mechanism of the host, and the function of acquired resistance is not, as might be expected, to counteract this virulence factor, but rather in some other unknown manner to permit the normal defensive substance to become effective.

This condensation represents the author's interpretation of the information presently available, and is subject to change as more evidence accumulates. It appears at least suggestive that no conventional antigen-antibody type of protective mechanism is involved in acquired resistance to the anthrax bacillus.

Practical procedures for vaccination.—Pasteur's original type of vaccine (29) is still employed for immunization of sheep and cattle. In this procedure use is made of two vaccines attenuated in varying degrees by cultivation at 42°C. The first lacks virulence for rabbits and guinea pigs, but is virulent for mice. The second lacks virulence only for rabbits, retaining its ability to infect guinea pigs and mice. Of vaccine 1, 0.1 ml. (for sheep) or 0.25 ml. (for cattle) of a 48-hr. culture is injected subcutaneously. The same amounts and routes are employed with the second vaccine 12 days later.

Spore vaccines (avirulent organisms) appear to be more effective than the Pasteur vaccine. Immunity tests carried out by the United States Bureau of Animal Industry have indicated that a single intradermal vaccination with such a vaccine produced rapid, dependable, and durable immunity in sheep (30).

Salsbery (31) has suggested vaccination with lesion edema fluid. In view of the results reported in experimental animals this would seem to be a feasible procedure.

It is recommended that annual vaccination be carried out in those districts where anthrax is known to be a potential threat (32).

Botulism

Active immunization against the toxins of *Clostridium botulinum* is limited to the production of antitoxins in animals. The

theoretical aspects of acquired resistance induced by this, as by other antigenic poisons, are relatively simple and no recent advances have occurred in this respect. During the past two or three years, however, certain of the toxins have been obtained in crystalline form, and the immunizing value of toxoids has also been discussed.

As is well known, five distinct antigenic types of toxin, all with the same pharmacologic activity, are produced by various strains of the bacillus. These are called A, B, C, D, and E. C is not entirely homogeneous; two varieties ($C\alpha$ and $C\beta$) are distinguished, with some cross reactivity in neutralization by antitoxin (33). Strains producing toxin types A and B occur in the United States and in England and are responsible for most cases of botulism in human beings. Both types of C occur in the United States, South Africa, and Australia; type D occurs in South Africa, and type E in Russia, and occasionally in this country.

Type A toxin was crystallized by Lamanna, McElroy & Eklund (34) and independently by Abrams, Kegeles & Hottle (35), in 1946. Putman (36) summarizes the properties of this material: it is of typical protein composition with respect to amino acid content, it is homogeneous in electophoresis, it sediments with a single boundary in the ultracentrifuge, and the molecular weight calculated from sedimentation and diffusion constants may be 900,000. There appear to be 19 constituent amino acids (37). This is the most potent toxin known, one mg. of nitrogen representing 220×10^6 mouse LD_{50}. Its behavior as a flocculating antigen has recently been described by Lamanna & Doak (39).

Type B toxin has been highly purified, but not crystallized, by Lamanna & Glassman (39). The purified material is a slightly colored simple protein, differing in chemical, physical and serological properties from the type A crystalline toxin. On the basis of N content its toxicity is only slightly less than that of type A, but on a molar basis it seems to be about 10 times less potent, since in molecular weight it appears to be about this much smaller than A.

Comparable purification or crystallization of other types of botulinum toxins has not been reported.

Toxoids and immunization.—The reduction of toxicity of botulinus toxin for purposes of immunization was first accomplished by Forssman (40) by means of heat. Weinberg & Goy (41)

first used formalinization for this purpose, with success. Formaldehyde inactivation of crystalline toxin A yields a toxoid which is homogeneous on electrophoresis and has negligible toxicity (36). Nigg *et al.* (42, 43) recently described the preparation of alum precipitated formalinized toxoid and the method for determining antigenicity in mice. Reames and co-workers (44) have employed fluid and alum-precipitated toxoids of types A and B for immunization of man. The data indicate that best results are obtained if a period of about two months is permitted to elapse between the first and a subsequent injection, as determined by the appearance of a presumptive protective level of 0.02 unit of antitoxin per ml. of serum. These results were essentially similar to those noted by Bennetts & Hall (45) earlier in a study of immunization of sheep with a similarly prepared type C toxoid.

Although the sporadic and infrequent occurrence of botulism in human beings makes this kind of prophylactic procedure an impractical one, information of this type might prove useful in time of war.

Cholera

Prophylactic vaccination against *Vibrio comma* has a long history and a confusing one, the latter in large part owing to the failure of this organism to reproduce the human type of infection in experimental animals. Much of the data concerning the value of vaccination and the usefulness of variously prepared vaccines have therefore been assessed from statistics derived from human populations, and such information is open to the various doubts inherent in this source. Burrows and his co-workers (46) recently surveyed this information and concluded that immunization of human beings probably results in some degree of effective protection. This apparently reasonable opinion plus the undoubted fact that protection against the bacterium (in an unnatural infection) in mice may be clearly demonstrated, will serve as the basis for the discussion which follows.

The protection-inducing factors.—The antigenic makeup of *V. comma* was reviewed by Linton in 1940 (47) and more recently by Burrows *et al.* (48). There is present in the organism a heat-stable, group specific somatic antigen as well as at least 12 other type specific antigens (48) in association with the group antigen in various strains. The H-antigens have no relation to acquired resistance (49).

The protection-inducing properties of vaccines and the passive protective powers of antisera may be studied in mice by employing the mucin method of infection described by Griffitts (50); intraperitoneal injection of a few organisms in 5 per cent mucin results in fatal infections. With this technic Burrows *et al.* (49) have attempted to assess the role of the somatic antigens in acquired resistance, by active and passive protection tests. The results were equivocal because although the agglutinating antibody (antisomatic) induced by boiled organisms appeared to take part in the resistance process, agglutinating and protective titers were not parallel [as seen also by earlier workers (51)]; and furthermore, antisera absorbed with somatic antigen retained protective ability. However, it was not possible to prepare protective antisera entirely lacking in somatic agglutinins. The authors could not therefore arrive at a conclusion regarding the protective qualities of anti-O antibody. Whatever its abilities, there appears to be another protective serum factor unrelated to it.

Burrows *et al.* (52, 53) have obtained from vibrios an endotoxin which is associated with, or is itself, a dialyzable phospholipid, and which is antigenic. Its antibody passively protects mice against bacteria (but not against the toxin itself) but such serum possesses no agglutinating properties for the bacterium. Perhaps this is the protective factor unadsorbable by somatic antigen; Burrows' writings do not clarify this point. Burnet & Stone (54) have discovered in filtrates of vibrio cultures a factor which damages specifically the epithelium of the guinea pig ileum *in vitro*. Singer, Wei & Hoa (55, 56) have confirmed the existence of this substance or substances and have studied it immunologically. It is antigenic, and sera produced in rabbit or man (56) by vaccination with killed bacilli also contain antibodies which neutralize this factor; they may or may not be the same as the antisomatic agglutinins. Several humoral responses may thus be induced by vaccination with the bacterium and may be concerned in acquired resistance, but the relationships have not been entirely clarified.

Relation between protection-inducing factors and virulence of the cholera vibrio.—Cholera infection in man seems to be of a most superficial kind, the vibrios remaining confined to the lumen of the intestine. At no time do they appear in the blood stream, and they are not seen to penetrate the intestinal submucosa. It appears likely that local damage to the intestinal mucosa

accounts for the essence of the disease; this is effected by bacterial autolysate—"endotoxins"—and results in loss of fluids and salts from the body. The importance of this to the disease process is reflected by the efficacy of simple parenteral replacement of water and salts in modifying its course and outcome [reduction of mortality from 50 to 70 per cent to 5 per cent (57)].

In view of the superficial nature of the infection, it seems doubtful that the somatic antigen functions as an "aggressive" factor here as it may in the case of the *Salmonellas*. Pathogenicity would appear to be linked rather with the cytoplasmic toxicity of the vibrio, a view which is supported by the observation of Burrows *et al.* (58) that the loss of virulence in strains which had been employed for the production of enteric infections in guinea pigs was coincident with a decline in the amounts of lipoidal endotoxin (52, 53) extractable from the cells.

The lipoidal endotoxin when tested upon intestinal strips *in vitro* is found (53) to increase considerably their permeability to fluids. Burnet & Stone's (54) culture filtrate factor acts upon the epithelium of the guinea pig ileum *in vitro*, causing this to shred and flake much in the same way as does the lining of the human intestine in cholera. One component of this toxic filtrate appears to be a "mucinase." What relation these toxic factors may have to each other is difficult to see; both substances appear to be part of the total cytoplasmic toxin of *V. comma*.

In view of the rather tentative status of our information regarding the factors responsible for inducing immunity, and those concerned in the pathogenicity of cholera vibrios, it is not possible to evaluate clearly their mutual relationships. The evidence at hand suggests, however, that such a relationship may exist, the resistance-inducing and the pathogenic properties of the organism being the same.

The mechanisms of acquired resistance to cholera.—As Burrows (49) has pointed out, the study of protection in the mouse to the intraperitoneal injection of vibros in mucin may have little relation to the situation obtaining in man because of the nature of the human disease. Consequently, he and his co-workers (58) have attempted a closer approach to the problem of acquired resistance in the human being by employing the guinea pig. Koch, in his original studies on etiology, successfully established enteric infection in this animal either by direct inoculation into the duo-

denum, or by oral inoculation following alkalinization with sodium carbonate and administration of opium. Burrows modified this procedure and obtained evidence that a true enteric infection, as indicated by multiplication of organisms in the intestinal tract and prolongation of the disease for several days, was produced. Little or no diarrhea occurred, but necropsy revealed shredding of intestinal epithelium.

Guinea pigs fed nonlethal doses of vibrios became resistant to several lethal doses of vibrios, and the numbers of bacteria in the feces were sharply reduced soon after ingestion. It was then found that specific antibody appeared in the intestinal lumen of animals following the first exposure to organisms, and that this occurred also following parenteral immunization with vaccine. This antibody was titrated as agglutinin, and its antibody character was demonstrated by the specificity of agglutinating activity, its absorbability by vibrios, its protective activity in mice, and by other criteria. Human beings were found also to secrete antibody into the intestinal tract following parenteral immunization with cholera or typhoid vaccine. This antibody extractable from feces is termed coproantibody by Burrows. Intestinal antibody against the dysentery bacillus was described by Davies (59) some years earlier.

The importance to acquired resistance of coproantibody is suggested by the observation of Burrows (58) that guinea pigs were not immune unless coproantibody were present at the time of challenge infection. In guinea pigs intestinal antibody could rarely be demonstrated for longer than 30 days, but in vaccinated human beings it may persist for about three months.

The mechanism of acquired defense in cholera may then depend to a large extent upon the appearance of antibodies in the lumen of the gut. These may be:

(*a*) Antisomatic antigen. Evidence for the protectiveness of this antibody is incomplete, but if it is involved in resistance it may function possibly as a lytic factor (with complement) since the vibrio is highly susceptible to this mode of lysis.

(*b*) A humoral protective factor, presumably antibody, which persists in serum after absorption with somatic antigen (49). The substance responsible for the evocation of this factor is not known, nor is its mode of activity appreciated; perhaps it is an

antiendotoxin. No information is as yet available concerning the appearance of this protective factor in the gut.

(*c*) An antiendotoxin against the epithelium-shredding factor of Burnet & Stone (54) and Singer *et al.* (55, 56). These antibodies also appear after vaccination with killed cells. Thus far we can only infer that they may appear in the intestinal lumen.

In addition, some form of intestinal tissue resistance may be acquired to the permeability factor described by Burrows (52, 53). The serum of animals immunized with this lipoidal endotoxin will not neutralize the toxic factor *in vitro* (though combining with it for various serologic tests). Furthermore, if normal intestinal strips are soaked in such serum, they are not protected from the permeability effects of the toxin. Yet strips removed from actively immunized animals are resistant to this toxic effect (60). Burrows suggests intracellular antibody within macrophages of the intestinal wall, but it is difficult to see why the location of antibody per se should determine its neutralizing ability.

These possible mechanisms embrace two essential effects; the first, a defense against the damaging endotoxins derived from bacterial bodies; the second, an antibacterial activity suggested by the characteristic disappearance of vibrios from the feces of patients within a few days after the cessation of the disease. Obviously, many gaps exist between the suggestiveness of current evidence and its establishment as fact.

Practical procedures for vaccination.—It seems apparent that any of a variety of procedures for producing a nonviable vaccine is equally satisfactory for vaccination purposes in man as judged by antibody responses and passive protection tests (55). Large numbers of bacilli are employed whatever the method of vaccine preparation, usually 8,000 to 10,000 million organisms per ml. One currently employed vaccine (61) consists of agar cultures killed by heat and preserved with 0.5 per cent phenol. An initial dose of 0.5 ml. is followed by a second dose of 1.0 ml. 7 to 10 days later. Booster injections of 1 ml. are recommended at six-month intervals. The opinion which dictates this frequent revaccination coincides with Burrows' (58) observations of the brief persistence of coproantibody following immunization. The Allied Forces vaccine and mode of administration is similar. Vaccine standardization is carried out by the mouse mucin test (51).

Diphtheria

The protection-inducing factor of Corynebacterium diphtheriae.—Diphtheria toxin was isolated in highly purified form by Eaton (62, 63) and by Pappenheimer (64) some years ago, and characterized as a protein of about 72,000 molecular weight. Recent work suggests that it may be the protein moiety of a respiratory enzyme of the bacillus—cytochrome-*b* (65). The fascinating evidence upon which this hypothesis rests reveals that toxin occurs in quantity only when insufficient iron is present in the medium to combine with it and porphyrin and thus complete the synthesis of the respiratory enzyme. The iron concentration optimal for maximum toxin production is thus low—far below that which exists in tissues (66).

Recently, Pillemer *et al.* (67, 68, 69) have prepared purified toxoid (formalinized) containing over 2,000 Lf per mg. of nitrogen by a process employing methanol precipitation at controlled pH, ionic strength, and temperature. An immunizing dose of 20 Lf of such material contains about 10 gamma of nitrogen. This toxoid can be precipitated with alum to yield a product of high antigenicity for human beings. The toxoid is stable at refrigerator temperature, and even at 37°C. for at least one month (69). Lawrence & Pappenheimer (70) isolated purified toxoid as well as an atoxic diphtherial protein (P) from culture filtrates and demonstrated their antigenic distinctiveness.

There is some evidence that acquired protection to diphtheria may depend upon a bacterial immunizing factor in addition to toxin. Phair (71) believes that adults who have had repeated exposures to the organism may, without developing sufficient levels of antitoxin, be able to suppress invasion by *C. diphtheriae* on the basis of an antibacterial mechanism. In view of the well-known rapidity and effectiveness of the anamnestic response to toxin, it would be difficult to obtain factual support for this supposition. However, Chason (72) and Gill (73) in Alabama, and Stebbins (74) in Kingston, New York, observed high incidences of Schick negativity in children without histories of clinical disease or of vaccination. Throat cultures revealed a very low occurrence of toxigenic bacilli but a much higher carrier rate of nontoxigenic organisms. This suggested that nontoxigenic organisms might be capable of inducing antibacterial resistance in human beings. It is difficult to see why nontoxigenic organisms

should be suspected of inducing a resistance which is measured by a test carried out with toxin. Nevertheless, Frobisher and his co-workers (75, 76) put this concept to test in rabbits by vaccination with nontoxigenic strains of bacilli, and found that such animals developed a localizing ability for subsequently injected toxin as well as for virulent bacilli. The absence of antitoxin in these animals was demonstrated. The authors could offer no immunological explanation for this acquired localizing ability for a heterologous antigen (toxin), but the recorded observations are forthright. Rosenau & Bailey (77) some years earlier had been unable to induce resistance in guinea pigs to toxin after prolonged vaccination with nontoxigenic diphtheria bacilli or with *C. hofmanni* or *C. xerosis*. The finding by Frobisher & Updyke (76) that the immunizing effect was obtainable with bacilli cultivated in a fresh pork medium, but not in veal medium, may possibly explain the failure of the earlier investigators who employed beef infusion broth.

What the immunizing factor in the bacillus may be is not known. Hewitt (78) has segregated about 50 serological types within the groups gravis, mitis, and intermedius by means of the agglutination reaction. Common antigens are shared by a number of types in all groups. The single suggestion known to the reviewer that antibacterial immunity may show some specificity on an antigenic basis is contained in the study by Orr-Ewing (79) of the effect of the leucocytes in whole blood upon the multiplication of various strains. He observed that the phagocytes of an individual infected with one type of organism were more active against it than against heterologous types.

It would be of scientific as well as practical interest to investigate further the possibility that acquired resistance to diphtheria may depend upon other factors in addition to antitoxin.

Relationship between protection-inducing factors and virulence.—The mode of pathogenic activity of diphtheria toxin is not yet known, but Pappenheimer (65) suggests that the toxin is part of a respiratory enzyme of the bacillus and may inhibit cytochrome-*b* in the host. Whether the diphtheria bacillus possesses pathogenic activities aside from that inherent in its exotoxin is questionable. The fact that only toxigenic bacilli cause disease may be a fortuitous circumstance. The ability of the organism to multiply in the host in the face of native defense processes suggests some kind

of aggressive property—but this may be simply an exotoxic effect since (*a*) the toxin damages leucocytes and (*b*) bacterial multiplication is restrained in animals in which the toxin is neutralized by antitoxin alone. Essentially, two kinds of observations have clouded the view that the pathogenicity of the diphtheria bacillus derives entirely from the activity of its soluble exotoxin. First, certain statistical reports indicate an inadequacy of toxoid immunization in modifying epidemics; secondly, the occurrence of cases of exceedingly severe disease, presumably caused by organisms of greater than ordinary virulence, and refractory to antitoxin therapy.

(*1*) *The inefficacy of toxoid immunization in modifying epidemics.*—Although the great majority of reports continue to support the effectiveness of toxoid immunization (80, 81, 82) disturbing exceptions have appeared. Eller & Frobisher (83) recorded an outbreak of diphtheria in Baltimore in 1944; of 142 children affected 63 per cent had history or health department record of immunization. The incidence of malignant ("bull-neck") or fatal disease was as high in the immunized as in the unimmunized group. Rendu (84) has reported a greater morbidity in the immunized in a study from Switzerland.

Several possible explanations are available to account for these failures without invoking the occurrence of extra-toxic pathogenic properties of the diphtheria bacillus. Toxoid preparations may vary considerably in their antigenic effectiveness, as recently commented upon by Bousfield (85). Individual differences in ability to respond to the antigenic stimulus have long been known (86 to 90). The procedure of routine immunization without subsequent check by Schick test may be misleading; Bullowa & Scannel (91) in New York found in 200 random instances 25 per cent failure to convert to negativity. And finally, as is fully appreciated, the negative Schick test is an arbitrary criterion of antitoxic immunity which covers a wide range of possible antitoxin contents in the circulating blood; at the lower levels consistent with a negative test clinical disease may occur (71, 87, 92). A thorough statistical analysis of the relationship of the occurrence and course of diphtheria with antitoxin titers determined at the onset of the disease has been made by Ipsen (93).

(*2*) *Evidence from strains of exceptional virulence producing disease refractory to antitoxin therapy.*—Perhaps a better defined approach

to the possibility of the existence of virulence factors in diphtheria bacilli aside from toxin is afforded by the study of strains causing malignant diphtheria. Some years ago Anderson *et al.* (94) subdivided diphtheria bacilli into groups on cultural grounds, and noted a decided correlation with the kind of disease from which isolation had been made. A so-called gravis group was frequently associated with malignant diphtheria, a group termed mitis most often was isolated from cases of ordinary severity, and intermediate forms of bacilli were also seen. Although European and British investigators continue to find such a general correlation [e.g. (95)], Frobisher (96) has been unable to confirm it in Baltimore and other parts of the United States. Nevertheless, he and others have observed malignant diphtheria, so that whatever the cultural characteristics of the strains isolated (frequently mitis by Frobisher) the essential fact remains that some diphtheria bacilli may cause exceptionally severe disease.

Conceivably, there may be several explanations for this: (*a*) that the highly virulent organisms elaborate an antigenically different toxin, or an additional toxic substance; (*b*) that such organisms produce simply more toxin than ordinary bacilli; or (*c*) that some pathogenic factor aside from toxicity accounts for the extreme virulence of these organisms.

There is little evidence to support the first point. Parish, Whatley & O'Brien (97), Povitsky, Eisner & Jackson (98), Zinnemann (99), and Hewitt (100) have established the antigenic identity of the diphtheria toxin produced by all strains. Nor has the question of an additional toxic substance (e.g., spreading factor) (101) received confirmatory support (102, 103).

As regards the second point, that highly virulent bacilli produce more toxin than less virulent organisms, Hewitt (100) has recently assessed a large number of strains of gravis, mitis, and intermedius bacilli for toxigenicity in culture and virulence in guinea pigs. There was marked parallelism in these regards, but there was no indication that gravis strains could, on the average, produce more toxin in culture than mitis strains. But that the situation in culture medium may be entirely different from that in a host has been strongly suggested by Mueller's (66) experiments in media containing various concentrations of iron. He found that the iron content of diphtheritic membranes was many times that optimal for toxin production *in vitro*. When he tested four strains

of bacilli for their ability to produce toxin in media containing large amounts of iron, only one, a gravis strain isolated from a case of malignant diphtheria, elaborated a considerable quantity in the highest iron-containing medium employed. This surpassed by almost fifteenfold the toxin production of the other three strains, and this despite the fact that at optimal iron concentrations another strain (Park 8) was far superior to the gravis strain in toxin production. Zinnemann (104) has extended these observations with more strains and with medium more closely approximating the composition of animal tissues. This evidence appears to provide a very likely lead to the answer of the increased virulence of certain strains of bacilli growing in the tissues.

Orr-Ewing (79) has approached the quantitative aspect of toxin production from another standpoint: the better ability of highly virulent strains to multiply in the tissues, hence to elaborate more toxin on the basis of larger numbers. Tests of susceptibility to phagocytosis in whole blood preparations revealed that most gravis strains resisted this more successfully that did mitis strains. This also argues inferentially for the possession by highly virulent organisms of a specific "virulence factor" in the nature of a leucocyte repellent. It was indicated in this report that an increased capacity for killing gravis organisms could be acquired as the result of infection with a gravis strain.

The third possibility suggests that increased virulence may depend upon factors other than toxin. Updyke & Frobisher (105) inquired whether another infectious agent may act synergistically with *C. diphtheriae* to produce malignant disease. Since streptococci of Group B had been isolated from several cases of malignant diphtheria in Baltimore (the diphtheria bacilli were mitis forms), this organism and 32 other species were employed in mice and rabbits in combination with diphtheria bacilli. Enhanced reactions were seen in some instances, but since all were controlled by administration of antitoxin, and since malignant diphtheria is not, the authors did not attach significance to the results.

In summary, diphtheria exotoxin itself is chiefly concerned in the pathogenicity of the bacillus, and significant variations in virulence of bacilli appear, from present evidence, to be owing to the capacity of the highly virulent strains to produce more toxin in the metabolic environment supplied by the tissues. More

evidence will be needed to permit judgment of other possible factors in virulence.

Mechanisms of acquired resistance to diphtheria.—The antitoxic type of immune response produced by the horse has been further characterized by Treffers, Heidelberger & Freund (106, 107, 108). Diphtheria antitoxin is produced by this animal following subcutaneous but not intravenous immunizing injections. The antibody is of a special type in that it flocculates in a narrow range of proportions with toxin, it occurs in the pseudoglobulin portion of the serum, and in the electrophoretic cell it migrates between the beta and gamma components (termed $beta_2$ or T component). If rabbit serum albumin is employed as antigen it induces the same kind of antibody response, and Pappenheimer (109) and Boyd & Hooker (110) had previously found that egg albumin and hemocyanin induce analogous antibodies in the horse. This contrasts with the response to pneumococcal nucleoprotein, pneumococcal S.S.S., and rabbit globulin, in which cases intravenous injection results in the appearance of antibody with broad flocculative reactivity, salting out as euglobulin and migrating predominately as γ-globulin. Ipsen (93), in discussing the relation of antitoxin level to onset and course of diphtheria, states that the occurrence of the disease in the face of high antitoxin titers may stem from the same sharply drawn neutralization limits seen in toxin-antitoxin mixtures *in vitro*.

Pappenheimer & Lawrence (111, 112) find a close relation between circulating antitoxin and the occurrence of a delayed type of allergic response to intracutaneously injected purified toxoid; so much so that sensitivity to toxoid is stated to be indicative of the presence of humoral antitoxin.

The recognition of the variability in significance of the negative Schick test has been described repeatedly over the years; some of the more recent expressions are contained in (93, 113).

As regards the possibility that antibacterial resistance may be of some importance in total protection against diphtheria, the work of Orr-Ewing (79) and of Frobisher and co-workers (75, 76) has been discussed above. Whether the partially effective antibacterial immunity induced with nontoxigenic bacilli by the latter investigators may be related to the differences in phagocytosis of strains observed by the former is not known. Possibly antibacterial

resistance may result in more effective phagocytosis of highly virulent organisms. Huang (114, 115) has described the presence of agglutinins in horse antitoxic sera employed for treatment, and believes that these may have been responsible for the rapid disappearance of a serologically homologous infecting organism from the throats of convalescent patients. Patients with heterologous bacilli of eight other types (115) maintained the carrier state for longer periods. Oddly enough, Ipsen (93) found in his statistical analysis that patients vaccinated with toxoid fared better in their illnesses than did the unvaccinated who had, as the result of natural exposure to toxigenic bacilli, the same initial antitoxin titer and a similar early clinical picture. It would be imagined that the latter group, with equal antitoxin titers acquired through spontaneous exposure to the organism, should have shown the benefit of antibacterial immunity as well if this is of importance to resistance.

Practical procedures for vaccination.—The recent literature concerning vaccination procedures is voluminous; only samples can be included here. Edsall (113) in a 1946 review stated that alum-precipitated toxoid is possibly slightly superior to fluid toxoid; this view is generally held. Alum-precipitated material possesses the disadvantage of provoking enhanced hypersensitive reactions as well as more frequent local reactions. However, the use of purified toxoid should eliminate many instances of hypersensitive reactivity to the bacillary constituents present in crude toxoid (112, 116). Lawrence & Pappenheimer's studies with purified toxoid and diphtherial protein P (70) revealed that Schick positive persons are predominately sensitive to the P substance (111) presumably as the result of previous spontaneous exposure to bacteria. Consequently, for these individuals who are most in need of immunization, the use of purified toxoid eliminates the major allergenic antigen. Most Schick negative individuals show the tuberculin-type of reactivity to purified toxoid, and in these persons reactions cannot be avoided. Since these individuals already possess a level of antitoxin however, very small "booster" doses may be employed, reliance being placed upon the repeatedly demonstrated (112, 113, 117) effectiveness of small doses of diphtheria toxin in inducing pronounced anamnestic responses. The Schick test dose may itself serve as an adequate booster stimulus.

In this way the occurrence of hypersensitive reactions may be minimized (112).

Purified toxoid in addition possesses a high level of antigenicity (69) and will no doubt soon supersede entirely the crude toxoids. Greenberg & Roblin (118) have described methods for standardizing the immunogenic values of toxoids.

Edsall (113) states that a minimum of two doses of alum-precipitated or three doses of fluid toxoid are necessary to produce an adequate and sustained immunity. Spacing of injections with either agent should be at least three weeks apart, preferably longer. (This corresponds to the experience with botulinus and gas gangrene toxoids in human beings.) Most evidence indicates that the subcutaneous route is best, although the intramuscular route has also been advised (119).

The duration of antitoxic immunity is variable, one group of workers (120) reporting that 34 per cent of children had reverted to Schick positive within five years after adequate immunization. The need for reimmunization is thus obvious, and the greater the efficacy of immunization programs in causing a decrease in carriers of toxigenic bacilli, the less the occurrence of spontaneous immunization and the larger the need for adequate artificial restimulation.

The widespread use of combined vaccines consisting of diphtheria and tetanus toxoids and *Hemophilus pertussis* possesses obvious advantages as well as some disadvantages which are discussed by Miller (119).

Bacillary Dysentery

There is no practical vaccination procedure for bacillary dysentery, but the subject is under active study and is of interest to a review concerned with the basic aspects of active immunization. A survey by Hardy & Watt (121) in 1944 revealed a higher incidence of *Shigella* infection (clinical cases and carriers) than is generally supposed to occur (11 per cent in New Mexico, 3 per cent in Georgia, 0.10 per cent in New York City). The carrier rate following clinical disease was found to be about 80 per cent with an average duration of 34 days after recovery. In all areas studied, the most probable etiologic diagnosis for endemic acute diarrhoea in older children and adults, especially in the warm

seasons, proved to be *Shigella paradysenteriae*; this was true also of enteritis in infants and young children in the south and southwestern parts of the United States. Under wartime conditions the problem becomes greatly magnified [Weil (122, 122a)]. The potential usefulness of a satisfactory vaccine is thus evident.

Protection-inducing factors in dysentery bacilli.—Two kinds of factors must be considered, according to the species of bacillus. *S. dysenteriae* elaborates—or more properly, releases upon autolysis—an antigenic toxin, a protein, which though not strictly speaking an "exotoxin" is often so termed because its poisonous properties are neutralizable by antibody, and because of its relative heat lability. A second factor occurs in all shigellae, although with different antigenic specificities, and is a toxic somatic (O) antigen of the "Boivin type," of common occurrence amongst the enteric bacilli (123, 124, 125). Their toxic properties are not appreciably neutralized by specific antibody and they are relatively heat-stable and so fall under the older concept of "endotoxins."

(*1*) *Shiga "exotoxin."*—This protein occurs in rough as well as smooth forms of *S. dysenteriae* and is released following autolysis or disruption of cells (126). Purification has been effected by Anderson, Brown & Macsween (127), Olitzki & Bichowsky (128) and Dubos & Geiger (129). It is best produced under highly aerobic conditions in a medium free of inorganic iron (129). Toxoid, prepared by formalinization, induces antitoxin formation in animals and human beings (127, 130), but no agglutinins for the bacillus (127). There is some disagreement as to the breadth of protection conferred upon animals by immunization with this toxin. Although it is generally opined that immunized animals are protected against the toxin itself (122, 129, 131 to 134), some investigators report that such immunity also protects against living virulent bacilli (129, 132, 134) while others (122, 131) have not observed this effect. Unfortunately for the clarification of this issue, in some cases (132, 134) toxin has been prepared from smooth bacilli, so that somatic antigen is probably included in the vaccine and may account for bacterial protection. However, Dubos & Geiger (129) prepared toxin from rough bacilli, precluding the presence of O antigen, and found the derived toxoid to activate protection in mice not only for the toxin and living Shiga bacilli, but for Flexner and Sonne organisms as well. No comment was offered regarding

the antigenic basis for this heterologity of resistance induced by the toxoid. This observation affords promise, not yet investigated, for a polyvalent immunization procedure for man.

(2) *Somatic antigens.*—The chemical and serological aspects of the somatic antigens of smooth phase shigellae are fully discussed in (125, 135 to 143) and the information is reviewed by Weil (122). These substances are complexes of polysaccharide, lipid, and polypeptide, and account for the "endotoxicity" of the bacilli, as well as for the induction of agglutinins and of protective antibodies as revealed by mouse protection tests (134, 139, 144, 145). The polysaccharide component, itself a hapten [although productive of antibodies in man and the mouse (139)], determines the antigenic specificity of the complex (146, 147). *S. paradysenteriae* (Flexner) is antigenically heterogeneous; the several systems of designation of types are discussed in (122, 122a, 148). The system proposed by Weil *et al.* (148, 149) establishes 19 types. These antigens are readily extractable by a variety of methods (refs. on chemical aspects above). *S. sonnei* has recently been reported to undergo phase variation with changes in the antigenic specificity of the somatic antigen (149a).

The antigenic activity of these complexes in stimulating the production of agglutinins and protective antibodies in mice and rabbits is unquestioned. Sera containing such antibodies confer protection passively. Perlman & Weil (147) found that the antibody induced by formol killed bacilli (*S. paradysenteriae* Type III) in rabbits could be absorbed with isolated O antigen to the extent of 97 per cent of its precipitative and 95 per cent of its mouse protective properties, and that the polysaccharide portion of the complex alone removed 90 per cent of the total precipitative and 77 per cent of protective antibody. In human beings also the somatic antigen induces agglutinins as well as protective substance demonstrable by tests in mice (134, 139, 146 to 148).

Despite this evidence for the identity of agglutinative and protective substance and their relation to the somatic antigen, some studies with the sera of vaccinated animals and human beings (150) and with human convalescent sera (151) have failed to establish this parallelism. Agglutinins may be absent in the presence of protective substance, or if both are present their relative values may be inconsistent. Morgan & Schütze (152) concerned

themselves with this question and obtained evidence that incomplete or univalent antibody, of a kind analogous to that encountered in anti-Rh human sera, may account for failure of agglutination. Sera from individuals who had been vaccinated with *S. dysenteriae* were studied by the technic of Coombs, Mourant & Race (153) and the titer of anti-Shiga antibody was in some instances amplified eighteenfold over that obtained by conventional titration. Parallel protection tests were not carried out, but the results obtained suggest an explanation for the failure of coincidence of agglutinative and protective titration values.

There are thus clearly two kinds of potential protection-inducing factors in the shigellae; the proteinaceous "exotoxin" of *S. dysenteriae*, and the somatic antigens ("endotoxins") of this as well as other species. Their ability to engender humoral protection demonstrable by animal tests is high; whether they may induce appreciable protection against the spontaneous disease in man will be discussed below.

Relationship of protection-inducing factors to virulence.—Information is available regarding the relationships of the factors described above to the pathogenesis of bacillary dysentery. The exotoxin of *S. dysenteriae* is referred to as a neurotoxin chiefly because of its effects in the rabbit, in which it produces paralytic and other nervous manifestations. Such phenomena appear to occur infrequently in man with dysentery caused by the Shiga bacillus; infants develop convulsions, nuchal rigidity, and other nervous manifestations more frequently than adults, but this may occur with other species than Shiga and probably is not necessarily related to the activity of this specific toxin. Steabben (131) reports that this toxin injected intracutaneously into rabbits causes changes in the intestinal mucosa (hemorrhagic patches) more marked than those in the nervous system, and that such enterotoxic activity is also dominant in man. Branham & Habel (154) observed enterotoxic effects also in experiments in monkeys; intravenous injections of small amounts of the toxin resulted in focal hemorrhages into many tissues, including the walls of the colon. Fed by mouth, however, insufficient absorption occurred through the intact mucosa to permit the toxic effects to become manifest. Dubos & Geiger (129) report that the accidental inhalation of powdered exotoxin by workers in the laboratory induced fever, headache, nausea and

diarrhea. Since this toxin is present in rough (avirulent) bacilli as well as smooth strains, whatever part it may have in the disease process resulting from Shiga infection is secondary in the sense that an organism with ability to produce infection (i.e., a smooth bacillus) is a preliminary requisite.

The somatic antigens probably have a dual role in pathogenesis. In the first place, only bacilli possessing such antigen can cause disease [although possible exceptions to this may occur (155)]. This may be accounted for by the ability of these somatic antigens to cause leucopenia (146) and to repel phagocytes (156), and other activities of an aggressive nature may be involved (146). In addition, they induce general toxicity following parenteral injection and cause pronounced enteritis, with petechiae or intense vascular congestion, in mice, guinea pigs, and rabbits (131, 157). Oddly enough, however, the ordinarily nonpathogenic enteric bacillus *E. coli* possesses O antigen with similar properties (157). It appears then that virulence of the dysentery bacilli depends upon more than the possession of the O antigen alone, though this is essential for virulence. What other properties of virulence there may be are not known. There is evidence from infectivity experiments in man that organisms with good somatic antigen content and with high virulence for the mouse are not necessarily of high virulence when ingested by human beings (158).

In summary then, the exotoxin of *S. dysenteriae* and the somatic antigens of shigellae generally appear to be capable of activating protective antibodies demonstrable by animal tests, and both factors may have a part in the pathogenesis of dysentery. The toxicity of the somatic antigen is said by Perlman & Goebel (159) and Tal & Olitzki (146) to reside in the protein portion of the complex. Other unknown factors are doubtless also concerned in the pathogenic activities of these bacilli.

The mechanism of resistance to bacillary dysentery.—Protective resistance in experimental animals at least appears to be directed against those factors which we have considered to be virulence factors, i.e., the exotoxin of *S. dysenteriae* and the somatic antigens of this and other species of *Shigella*. The difficulty of assessment in relation to the human being is similar to that discussed in regard to cholera; namely, whether evidence of protection obtained in animals challenged by the intraperitoneal injection of bacilli in

mucin, or by intracerebral inoculation, may validly be transposed to the spontaneous disease in man, for in their enteric location organisms may be inaccessible to humoral immune bodies. There is, in fact, some question as to whether resistance to bacillary dysentery occurs following an attack of the disease. Hardy & Watt (121) found in institutional inmates that a preceding clinical infection provided a degree of protection against subsequent clinical attacks with a homologous *Shigella*, but little protection against subclinical infection with such a strain. No cross protection was seen at all.

From the standpoint of antibody protection against an organism established in the lumen of the bowel, the evidence presented by Davies (59) and by Burrows *et al.* (58) of the existence of coproantibody (see section on CHOLERA) may be of utmost pertinence. If antibody may indeed be excreted into the gut, it is conceivable that Shiga antitoxin would neutralize the toxin of this bacillus directly. The antisomatic antibodies could not correspondingly neutralize the poisonous properties of O antigen, for the toxicity of these substances is not appreciably decreased by combination with antibody, but if union with this antigen in the intact bacilli should encourage phagocytosis then organisms might be eliminated before sufficient multiplication had ensued to permit the accumulation of these toxic substances in harmful amounts. It seems probable that this mechanism of protection, if it is a true picture, is not as effective as that in cholera, for in the latter disease the resistance which results from an attack rapidly clears organisms from the intestine. Recovery from dysentery on the other hand is commonly followed by a carrier state of variable duration (121). May this be because an antibody-complement lytic mechanism is very effective in the destruction of *V. comma*, and is of much less, if any, significance in the case of the shigellae?

The broad protection reported by Dubos & Geiger (129) to follow immunization of mice with the toxoid derived from *S. dysenteriae* alone, effective against Shiga, Flexner, and Sonne organisms and demonstrable by a greatly enhanced bactericidal activity of the sera upon these various shigellae *in vitro*, cannot be assessed from the standpoint of mechanism at this time. Presumably humoral antibody is concerned, although from the standpoint of antigenic specificity this view is difficult to reconcile with

the facts presently established. If this is an instance of conventional antibody protection, the same considerations discussed above would probably apply here also.

Vaccination procedures for bacillary dysentery.—The several considerations which will determine the eventual establishment of a satisfactory vaccine for bacillary dysentery are these: the preparation of a vaccinating agent of sufficiently broad antigenic properties to cover the most frequent etiologic species and types; the reduction of the toxicity of such vaccines to the point of practical usefulness; and the demonstration that they can afford protection against the enteric disease in man. Recent evidence is available on these points.

(*1*) *Vaccines of broad antigenic properties.*—The most encouraging lead with regard to breadth of protection is provided by the work of Dubos & Geiger (129) described above, with Shiga toxoid. Less inclusive, but promising, has been the use by Perlman, Binkley & Goebel (139) of the somatic antigen of *S. paradysenteriae* Type V (Andrewes and Inman). In human beings this evoked antibodies cross reacting with Type Z organisms, and it was pointed out that the V, Z, VZ, and Y types are closely related serologically. These authors suggest that a mixed vaccine containing Types V and W of *S. paradysenteriae*, *Shigella* sp. Newcastle, and *Shigella* sp. Sonne would anticipate the greatest number of enteric infections. Weil (122) summarizes data indicating that where dual type antigens occur cross protection against organisms containing one or the other antigen is good.

(*2*) *Detoxification of vaccines.*—The detoxification of Shiga exotoxin may be accomplished by formalinization (129, 130) or by ultraviolet irradiation (132). Toxicity for man is not entirely eliminated by either method.

More difficulty is involved in reducing the toxicity of somatic antigens, or of whole bacillary vaccines. Halbert *et al.* (160, 161) employed both kinds of antigens in saline-in-oil emulsions, and found a considerable reduction of toxicity along with enhancement and prolongation of antibody responses in animals. However, later work (162) with similar vaccines in human beings proved disappointing in respect to agglutinating and mouse protective properties of the sera. The authors' suggestion that too few organisms may have been employed (1.2 billion) finds support in a

study by Cooper, Tepper & Keller (150) which appeared some months later. Total vaccinating doses employed in children ranged between 15 and 30 billion organisms by the subcutaneous route, and appreciable agglutinative and mouse protective responses occurred. It appears that *S. paradysenteriae* vaccines will of necessity contain large concentrations of bacillary material. Vaccination by the intravenous route resulted in a considerable response to relatively small amounts of vaccine (10 million organisms total), but the severe systemic reactions evoked were outside the pale of practicality.

Ultraviolet irradiation has been suggested for the detoxification of somatic antigens. Goebel, Binkley & Perlman (140) concluded that the reduction of toxicity is so closely paralleled by loss of antigenicity as to make this an unpromising approach, but Barnes *et al.* (163) have been more optimistic about this method or a variant of it. Treffers (164, 165) acetylated O antigen of *S. dysenteriae* with acetic anhydride and observed a sixtyfold reduction of toxicity for mice and rabbits with retention of ability to induce protection, but not agglutinins. A variety of other chemical procedures have been employed (140) with varying degrees of success. The problem of securing a favorable antigenicity/toxicity ratio is still an open one, though probably not far from a conclusion sufficiently favorable to permit the practice of routine vaccination in human beings wherever indicated.

(*3*) *The demonstration that dysentery vaccines may afford active protection to man.*—As might be expected, reports of the efficacy of vaccines in the protection of man run the gamut from high optimism to disappointment [Weil (122)]. A well-controlled human experiment has been described by Shaughnessy *et al.* (158) wherein a group of 53 adults was immunized with heat or ultraviolet killed vaccines of mixed types of *S. paradysenteriae*, and was subsequently challenged by the oral administration of living bacilli of one of the types represented in the vaccine. Clinical dysentery occurred as extensively in the vaccinated groups as in a control unvaccinated group of 30 individuals (over 60 per cent incidence). It appears to the reviewer that this study need not be too pessimistically interpreted for the reasons: first, that the total dose of vaccine employed contained 2 billion organisms of the type subsequently employed to test resistance, and as indicated by later data

(150, 162) this is probably insufficient for the proper immunization of human beings; second, the immunizing strain against which challenge was carried out was highly virulent for mice but, as later found, not for man, and homologous virulence may be an essential characteristic for a successful vaccine. Other suggestions for improvement in experimental conditions, such as wider spacing of immunizing injections, were also made by the authors.

Gas Gangrene

Acquired immunity in gas gangrene is considered here from the standpoint of those saccharolytic species of *Clostridium* which are primarily concerned in its etiology. With some justification there could be included discussions of the proteolytic species which often take part in the disease without, however, being able to initiate it—and of such other organisms, as the pyogenic cocci, the presence of which in the tissues may greatly facilitate the progress of the clostridia themselves.

The pathogenic species of the genus *Clostridium* to be discussed include *C. perfringens* (Type A), *C. septicum*, *C. novyi*, and to an extent limited by the information available, *C. histolyticum* and *C. bifermentans*. These, singly or in combination, account for the majority of the cases of gas gangrene.

The protection-inducing factors.—Reports are scant on the question of bacterial factors concerned in protective immunization, but what literature exists indicates that resistance cannot be induced by bacterial cells alone (166), although of course these stimulate antibody production. The reason for this ineffectiveness is not clear; it may be suggested that since the tissue in which these organisms usually initiate infection is damaged or dead, blood-borne protective substances may not reach them (167). However, in a similar clostridial disease of cattle, blackquarter or blackleg, caused principally by *C. chauvoei*, the prophylactic vaccine employed consists of formalin treated cultures, and resistance is thought to be both antibacterial and antitoxic (168). In view of the fact that this vaccine has been of practical usefulness for many years, it is doubtful that much critical inquiry has been directed toward these points, however.

The diffusible toxins are able to induce resistance, to the toxins themselves and to the progression of bacilli. The toxins of the

clostridia are now fairly well known. *C. perfringens* of Type A produces a lecithinase (169) referred to as alpha toxin which is hemolytic, necrotizing to cells, and lethal following intravenous injection. A second enzyme, collagenase, is proteolytic and destroys collagen (170); this is termed kappa toxin by Oakley *et al.* (171, 171a). Theta toxin is an oxygen labile hemolysin, chemically and immunologically related to similar labile hemolysins of other genera of bacteria (172). Finally, hyaluronidase is produced by *perfringens* and other species (173).

All the saccharolytic (i.e., primarily pathogenic) clostridia produce diffusible toxins with analogous effects. Thus *C. septicum* toxin causes hemolysis and local necrosis, and is lethal when injected intravenously. So far as is known one toxin possesses all these activities. The toxin is relatively weak as measured by concentration in culture filtrates, but potent in end results (174). *C. novyi* elaborates a toxin with similar activities, but more powerful from the quantitative standpoint than that of *C. septicum;* here there are multiple toxic factors, Oakley *et al.* (175) having described three components of Type A (gas gangrene producing) bacilli, including a lecithinase with necrotizing properties, an hemolysin, and a lethal toxin. *C. bifermentans* produces a lecithinase antigenically related to that of *C. perfringens* (176) but with only slight toxicity to the tissues of guinea pigs. *C. histolyticum* is a member of the proteolytic group of bacilli not always indicted of a primary role in the etiology of gas gangrene, but British reports from the Middle East during the recent war indicate that it may have some importance in this regard (177). A lecithinase is produced by this organism (175, 178), as is collagenase (179).

The systemic activities of these various toxins have been investigated. Zamecnik, Nathanson & Aub have described these for *C. perfringens* (180) and for *C. novyi* (181), and Pasternack & Bengston (182) for *C. septicum*. *C. perfringens* toxic filtrate produces gross intravascular hemolysis and fall in blood pressure; *C. novyi* toxin acts principally upon the peripheral vascular system; and with *C. septicum* filtrate cardiotoxic and nephrotoxic properties predominate.

Some information regarding successful immunization against gas gangrene by the use of toxoids has been available for years, but a great deal was added during the latter years of World War

II and through 1947. The relative inactivity in this work since that time probably owes to the fact that additional information will have to come from field tests in human beings. Danielson (177) has provided a good summary of this recent work carried out by groups in America with the toxoids of *perfringens*, *novyi*, and *septicum*. Stewart (183), Robertson & Keppie (184), Altemeier *et al.* (185) and Tytell *et al.* (186) have all demonstrated in various laboratory animals that immunization with the toxoid of *C. perfringens* induces protective resistance to living organisms. The same findings have been reported for the toxoid of *C. novyi* (184, 186). In the case of *C. septicum*, Craddock & Parish (174) have demonstrated the prophylactic protection against living organisms afforded by antitoxic serum.

Comparative tests of protection against *C. perfringens* in various species of animals are well illustrated by the work of Tytell *et al.* (186). In guinea pigs and dogs, serum antitoxic values (based upon antilecithinase activity) of 0.10 unit or more per ml. were found to correlate with dependable protection against living organisms. Additional evidence of the same nature is presented in (177). As for the human being, it is possible with alum-precipitated toxoids of *perfringens*, *novyi*, and *septicum*, alone (186, 187, 188) or combined (177, 186, 188, 189), to effect antitoxic responses in practically all subjects to a level above that found protective for animals.

In the case of *C. perfringens* the toxic components are sufficiently well known so that it has been possible to assess the capacity of the individual toxic factors to induce resistance. Such investigations have been carried out chiefly by Evans (190, 191, 192) by the use of passive protection tests. Antitoxic sera prepared in horses, and containing individual antitoxins almost exclusively, were tested for their abilities to protect guinea pigs from intramuscularly injected virulent bacilli. Whereas antibodies induced by the theta toxin (190), hyaluronidase (191), and collagenase (192) have been devoid of protective ability in these experiments, the antilecithinase (alpha antitoxin) has proved highly effective (190, 191, 192).

Relation between the protection-inducing factors and virulence of clostridia.—The toxins of the clostridia are apparently closely related to their pathogenic abilities, both in the sense of permitting

the bacteria to establish infections, as well as in accounting for much of the local and systemic damage which occurs in the disease (166, 181, 182). Possibly bacterial factors may also determine the ability of these organisms to establish themselves in the tissues, but this possibility has never been clearly delineated. The "virulence function" of *C. perfringens* toxin was well shown by De-Kruif & Bollman (166) in 1917. Large numbers of washed bacilli were required to initiate infection in guinea pigs, but much smaller numbers sufficed when injected along with toxic filtrate, the infectiousness of washed organisms being thereby increased ten thousandfold. Later efforts have been made to find which of the toxic components of *C. perfringens* may be most concerned in this effect. McClean (173) concluded from a study of hyaluronidase production by clostridia that in general those organisms capable of dramatic tissue invasion are those which elaborate this particular factor, and (193) that the majority of strains isolated from clinical cases of gas gangrene produce it. Kass *et al.* (194) interested themselves in this enzyme as well as the alpha toxin (lecithinase). Ninety-four strains isolated from human and animal feces and from soil were tested for hyaluronidase and lecithinase production, and for virulence in mice. The results indicate a high correlation of lecithinase production with virulence, and a lower correlation between hyaluronidase and virulence. Of the virulent strains, 83 per cent produced lecithinase while 54 per cent produced hyaluronidase; most of the latter were lecithinase producers also. However, only about one-half the lecithinase producing strains were virulent by the method of test used here, so that ability to produce this toxin does not necessarily indicate infectiousness. Furthermore one-fourth of the strains producing hyaluronidase alone were virulent, as were the same proportions of strains producing neither enzyme. Despite the good correlation of lecithinase with virulence, there are obviously other factors which these experiments do not clarify. Evans (195) carried out a similar study, extending his survey to the theta hemolysin as well as lecithinase and hyaluronidase. Thirty strains were investigated for production of these substances and for ability to infect guinea pigs on intramuscular injection. The correlation of virulence with lecithinase was again high, but again some strains (2 of 21) producing higher concentrations of this enzyme were avirulent. Whether or not strains pro-

duced hyaluronidase or theta toxin did not coincide with virulence. The collagenase responsible for pulping of muscle has not been similarly studied for its possible role in the genesis of gas gangrene infection. Evidence regarding this substance is presumptive, based on the inability of anticollagenase immune serum to hinder the initiation or progression of experimental gangrene (192). The reviewer terms this evidence presumptive because, as seen in the case of the anthrax bacillus, antibodies formed against a virulence factor need not necessarily constitute the protective mechanism in acquired immunity. It has been pointed out by Evans (179) that collagenase may contribute to the disease picture, however, by producing extensive muscle destruction. It might be revealing to carry out experiments with individual toxic components of *C. perfringens* of the type performed by DeKruif & Bollman (166) with the whole toxic filtrate.

Thus, the evidence for the importance of the lecithinase of *C. perfringens* in relation to its virulence is good, and this substance also is of most importance in induction of resistance. What other factors may be involved in the infectious ability of *C. perfringens*, as suggested by the fact of incomplete correlation with lecithinase production, are unknown. These may be diffusible factors also, or may depend upon constituents or properties of the bacterial bodies themselves. Since the production of toxin by clostridia may be limited by the iron content of the medium (196), it seems possible that this, or perhaps some other environmental factor, may mask the toxigenic properties of some strains as determined in culture. As in the case of *Corynebacterium diphtheriae* (loc. cit.), this may also suggest varying abilities of strains to elaborate toxin in the presence of the large quantities of iron present in the tissues.

On the basis of the information presently available we may envision a qualified picture of the mechanism of gas gangrene infection as was described by DeKruif & Bollman (166) over 30 years ago. These anaerobic bacilli initially multiply in devitalized tissue. The diffusible toxins emanating from such a locus damage or destroy neighboring viable tissue, and the bacilli follow in the wake of this injury. Fermentation of tissue carbohydrate results in gas formation, and the characteristic local picture of gas gangrene is produced. Meanwhile, general intoxication occurs as well [e.g. (180, 181, 182)]. Hyaluronidase may aid the diffusion of the

other toxic factors (180), and collagenase may add its characteristic activity to create the pulped muscle appearance generally seen in gangrene. This evidence is most complete for *C. perfringens*, but probably applies as well to those other clostridia which are able to initiate gas gangrene infections. Other factors, as yet unknown, also appear to determine the ability of clostridia to establish infection.

The mechanism of acquired resistance to gas gangrene.—The fact that the toxin of *C. perfringens* chiefly responsible for virulence also induces protective resistance to the organism permits the formulation of a simple concept of the mechanism through which acquired resistance is effective. In fact, experimental findings exceed the expectations deriving from studies such as those described above, for even with those virulent strains of *C. perfringens* which do not appear to elaborate lecithinase as determined by the *in vitro* methods employed, antitoxin against this factor protects animals against intramuscularly injected bacteria (195). Analogously, *C. septicum* antitoxin protects animals against infection with this organism (174), and the same is true for *C. novyi* (186).

The mechanics of resistance appear then to be straightforward. If a sufficient level of antitoxin is present the necrotizing activity of toxin is neutralized, the bacteria cannot spread from the point of entry because additional devitalized tissue is not made available, and the infection terminates. Recently, Keppie & Macfarlane (197) inquired whether antilecithinase may be present in myosin and other muscle proteins of animals immunized with *C. perfringens* toxoid, as well as in the circulating fluids. No evidence of "muscle immunity" was found.

Practical procedure for vaccination.—The need for a dependable prophylactic vaccine for military personnel in wartime is adequately established. The average incidence of gas gangrene in several reported groups of wounded soldiers during the last war was 1.2 per cent, and average mortality has ranged between 22 and 70 per cent (177). Therapy, which has depended chiefly upon surgical intervention and the administration of antitoxin, has limitations, especially with regard to time (174, 198). Since established gas gangrene may frequently involve infection with other organisms such as pyogenic cocci, effective treatment is further complicated.

Reports on vaccination which have appeared since 1945 indicate that the efforts instigated by the recent war have brought forth a multiple-toxoid vaccine (*C. perfringens*, *C. septicum*, and *C. novyi*) which, there is every reason to expect, will provide satisfactory resistance to gas gangrene in human beings. A previous obstacle, the lack of antigenic potency of toxoid preparations (199), has been overcome by newer cultural methods (196, 199 to 202) which furnish higher yields of toxin, and by chemical procedures for concentration (200). But even without these advantages it appears that the proper number and spacing of injections of alum-precipitated toxoids can engender protective levels of antitoxin in human beings (as judged by challenge tests in animals). It has been observed by several groups of investigators (177, 186, 188, 189) that if human beings are treated with three injections of toxoid, the last dose four to ten months after the first two, protective levels of antitoxin appear in almost 100 per cent of individuals treated, and these levels persist for seven to ten months. Adams (189) with perfringens and novyi toxoids demonstrated that even if relatively weak preparations were employed, alum precipitation and a long interval between the second and third doses resulted in satisfactory responses in at least 90 per cent of subjects. Mixed vaccines containing the toxoids of perfringens, septicum, and novyi are entirely satisfactory as antigenic stimuli (177, 186, 188, 189).

The common experience with regard to local and systemic reactions to these toxoids has been favorable; these do not exceed the reactions commonly seen with such established immunizing agents as tetanus and diphtheria toxoids.

General Comment

The half-dozen diseases treated here, randomly selected, nevertheless sample the variable natures of the factors involved in acquired resistance, and reflect the possibilities which, after 70 years of immunologic efforts, still remain for the extension of practical immunization procedures. The large step forward which resulted from the recognition of the chemical nature of the protection-inducing antigens of the pneumococcus and other organisms may be succeeded by another step dependent upon the recognition and isolation of substances which cannot so graphically be assigned a place in the structure of the cell, and which may engender resist-

ance on a somewhat different basis than the conventionally accepted antibody response. Even such less spectacular studies as those concerned with methods of concentrating antigens, and the suitable scheduling of immunizing doses, have much to contribute to fundamental information as well as to the development of useful immunizing procedures.

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CHEMISTRY OF TOXINS

By Louis Pillemer and Kenneth C. Robbins[1]
Institute of Pathology, Western Reserve University, Cleveland 6, Ohio

Bacteria contain or liberate toxic substances which are largely responsible for the clinical manifestations of infectious diseases. The chemist, physicist, and microbiologist have attempted to elucidate the physicochemical nature of toxins and to solve the riddle of their mode of action. Close co-operation among these workers, each applying his specialized discipline, should ultimately lead to increased knowledge of these bacterial poisons.

It is the purpose of this review to summarize existing knowledge of the chemical and physical nature of certain toxins and of their mode of action. On the whole, this report will be limited closely to recent work of general interest. No attempt has been made to cover every paper pertinent to this subject. Several excellent reviews (1 to 4) which cite adequate reference to all phases of the field have appeared since Eaton (5) summarized the literature.

Methods of Purification

With increasing knowledge of the chemistry of proteins, more interest is being shown in separating toxins from bacterial filtrates or extracts. Isolation in a pure state is imperative for a true understanding of the nature of toxins. Thus, once sufficiently pure toxins are available as concentrates, their use in medicine, biology, and chemistry will be manifold. Another compelling motive behind the purification of toxins has been the development of purified toxoids for use in immunization (6 to 9). The removal of destructive enzymes, unstable impurities, and allergenic impurities has greatly decreased the disadvantages of toxoids and should lead to an increased use of these agents for the prevention of infectious diseases.

Bacterial toxins appear to be proteins or protein complexes and, therefore, the chemistry of toxins is the chemistry of proteins. Toxins are irreversibly altered by heat, strong acids or alkalies,

[1] The authors are indebted to Dr. B. Cinader for the many contributions he has made to this manuscript. We wish to thank Ellen Welsh for her aid in the preparation of this review for publication.

and other denaturing agents. Thus, only certain procedures may be employed to fractionate and purify these substances without change. The most successful procedures are based on solubility differences between proteins, and the chemistry of toxin purification is essentially the chemistry of protein solubility.

Protein solubility is influenced by many factors, the control of which has formed the basis of protein fractionation. The number of conditions under accurate control which can be varied will influence greatly the precision with which the separation of these proteins can be carried out. Several methods have been employed for the purification of toxins. Most of them have utilized large amounts of salt to reduce the solubility either of the toxin or of impurities. Differences in the solubility of proteins in concentrated salt solutions are not great and are principally a function of the size of the protein molecule (10, 11). On the other hand, the solubility of proteins in dilute salt solutions is often very large and is indeed intensified in low dielectric media (10, 11). Such solubility phenomena appear to be related to the specific electrical properties of each protein (10, 11) and are, in fact, the basis for the alcohol methods. Salting-out procedures for the most part are largely based on the work of Denis and others. Although such empirical methods have yielded satisfactory preparations, it appears that they leave much to be desired and that the problem of purifying toxins should be solved by the employment of precise physicochemical methods.

The purification of toxins offers many difficulties, arising partly from the fact that toxins constitute a relatively small proportion of a heterogeneous mixture and partly from the fact that toxins are labile. In addition, it is necessary to work with large volumes of bacterial culture or bacterial filtrates to obtain a sufficient quantity of the final purified substance. Sterile culture filtrates of growing bacteria or disintegrated bacterial cells are employed as the source of toxin. The production of exotoxins on media of defined composition (2, 12 to 15) containing nutrients of low molecular weight has aided greatly in the purification of toxins. It should be noted that the problems encountered in the purification of these labile substances would have been greatly complicated without these media, and perhaps, the actual isolations reported below would have been impossible. The purification of toxins presents other difficulties such as their affinity for surfaces such as glass, etc. (6, 16). Finally,

several closely related proteins are always present which can only be separated by precise variations in the conditions for fractionation. Using some of the principles employed by Mellanby (17), Hardy & Gardiner (18), Felton (19), and Wu (20), Cohn and his associates (21, 22, 23) have developed a system for the fractionation of proteins in ethanol-water mixtures at low salt concentrations and low temperature. This process was successfully utilized for the fractionation of blood plasma. As pointed out by these workers, the denaturing effect of alcohol is minimized at the low temperatures employed. Dialysis, which is a major objection to neutral salt precipitation, is unnecessary in the process. The influence of pH, ionic strength, and protein concentration in this system of fractionation has been described (11). In studies on the purification of toxins (6, 24 to 31) methanol has been found preferable to ethanol as the organic precipitating agent. It was noted among the toxins studied that ethanol caused changes in the toxin molecule which was indicated by a marked prolongation of flocculation time. Clinical objections to the use of methanol are minimized because the volatile methanol is removed from the proteins by freezing the mixture and then removing both the water and the alcohol at reduced pressure.

For the most part, the influence of pH, alcohol concentration, temperature, and protein concentration on the solubility of toxins is similar to that described for plasma proteins (23). The precise balance between the charged condition of proteins (which is determined by the pH of the mixture) and the methanol concentration plays the major role in the separation of toxin from bacterial proteins and culture medium constituents. Protein concentration is adjusted to allow protective stabilization of the toxin molecules and to allow interplays by virtue of protein-protein interaction, and of their dipolar activities. Furthermore, the conditions for the resolution or extraction of precipitates must be carefully ascertained and controlled. The adjustment of five independent variables permits the attainment of a large number of possible experimental conditions for the separation of the desired proteins.

The solubility of the components of a protein system varies as a function of pH at constant ionic strength and constant methanol concentration, or as a function of methanol concentration at constant pH and constant ionic strength. Other sets of experimental values can be obtained as functions of temperature, ionic strength,

or protein concentration at constant methanol concentration or pH. Each variable is adjusted at any one step in order to obtain the highest yield and greatest purity without deleterious changes in the toxin molecules.

The conditions for removal of impurities at any one step in the fractionation are specific for that step. The conditions required for the separation of a particular toxin will not necessarily apply to another toxin. Such investigations involve the laborious task of physicochemical, analytical, and immunological control and do not offer any easy or simple application to the purification of all protein systems. The precision of the method, the ease in obtaining highly purified products once the exact method has been established, and the reproducibility of results make it the method of choice in the fractionation of functional proteins.

Criteria of Purity

It is apparent that bacteria which produce toxins are not entirely identical and it may be assumed that each bacterium will not produce uniform toxin molecules. Therefore, it is doubtful whether any toxin will eventually prove to be entirely homogeneous with respect to all chemical and physcial criteria. However, the commonly accepted criteria of purity of toxins are the same as for other proteins; that is, constancy of solubility, constancy of chemical composition, and homogeneity of physical properties. Since the toxin has measurable biological activity, a correlation must exist between the above properties and all the biological functions of the protein. It is imperative that the conditions employed in physical characterizations should be within the physiological range. This will allow a greater degree of correlation between the physical and chemical data and biological function of the toxin. Furthermore, it appears necessary to carry out all physical measurements on freshly prepared toxins. Identical samples should be employed for the comparison of biological activity with chemical and physical data, and these measurements should be made simultaneously. The recognition of the possibility that toxins may easily change upon standing should yield more precise data on these substances.

The biological functions of toxins serve not only as convenient measures of the degree of purification achieved during fractionation but also as a most important tool in observing early changes

(denaturation) in the toxin molecule. Physicochemical analysis may often reveal gross changes in the protein such as aggregation, degradation, or changes in asymmetry. However, none of the available physicochemical techniques are as sensitive or specific as are measurements of biological function. For example, in the case of toxins which flocculate with antitoxin the time required for flocculation (Kf) is a sensitive indicator of early denaturation. In fact, long before any physical or chemical change can be noted, damage to the molecule may be observed in a slight prolongation of the Kf. Furthermore, it is possible that the lethal activity of a toxin may be altered long before any physical or chemical change is observed.

Tetanal Toxin

Tetanal toxin (spasmin) has been isolated and crystallized (24, 26) using methanol as the organic precipitating agent under controlled conditions of pH, ionic strength, protein concentration, and temperature. The methods have been described in detail elsewhere (26). Depending upon the limits of solubility of the protein and of the impurities, the nature of the impurities, and the convenience of experimental conditions, the toxin was either precipitated or maintained in solution during the removal of impurities. Six steps were necessary to purify the toxin sufficiently to allow crystallization. The toxin is precipitated from the culture filtrate at pH 5.15 in 40 per cent methanol at −5°C. It is reprecipitated at pH 5.5 in 15 per cent methanol, ionic strength of 0.025 at −3°C. Impurities can be removed as a precipitate in the next step at pH 4.0 in 7.5 per cent methanol, ionic strength of 0.075 at −3°C. After the removal of these impurities, the methanol concentration can be increased to 30 per cent at −5°C. resulting in the further precipitation of other impurities with 90 per cent of the toxin remaining soluble. The toxin is precipitated from this solution at pH 5.4 in 10 per cent methanol, ionic strength of 0.02 at −5°C. The final step before crystallization which gives almost pure toxin is the reprecipitation at pH 4.9, ionic strength of 0.01 at 0°C. The yield at this step is 50 to 70 per cent. Crystallization was accomplished by two means. In very dilute solution the toxin will crystallize out of solution between pH 4.5 and 5.4. This procedure involves the handling of large volumes of material and is time consuming. A second more practical set of conditions for the crystallization of tetanal toxin has been established. Highly

purified tetanal toxin crystallizes from a 1.0 per cent protein solution at pH 6.0 in 20 per cent methanol, ionic strength of 0.02 at $-5°C$. The crystals can be redissolved in 0.15 *M* sodium acetate and recrystallized under the conditions stated above. The final product was dissolved either in 0.15 *M* sodium chloride at pH 5.5 or, for greater stability, in 0.3 *M* glycine at pH 5.8.

Crystalline tetanal toxin in a 1.0 per cent solution is pale yellow. It is very stable in the presence of dipolar ions between pH 5.0 and 6.0. The crystalline toxin gives the usual protein reactions and contains 15.7 per cent nitrogen, 1.0 per cent sulfur, less than 0.1 per cent phosphorus, and is entirely lacking in carbohydrate. The percentages of 13 amino acids in this protein have been determined (32). Tetanal toxin contains the same amino acids as diphtherial toxin and botulinum toxin, Type A, but in different proportion. High percentages of aspartic acid and isoleucine were found as compared to other simple proteins. It is of interest that the aspartic acid is about 50 per cent greater than the glutamic acid and that the percentage of isoleucine exceeds that of leucine. These relationships are similar in botulinum toxin, Type A (33), but are reversed in most other proteins of known composition. Whether or not the similarity of the amino acid content of these two toxins is related to their specific action upon nervous tissue remains to be determined. On the basis of the afore-mentioned results, tetanal toxin contains a number of amino acid residues per mole ranging from 3 for tryptophane to 47 for glutamic acid, 48 for isoleucine, and 76 for aspartic acid. The minimum molecular weight of tetanal toxin was estimated to be about 67,000.

Crystalline tetanal toxin is electrophoretically homogeneous with a mobility of 2.8×10^{-5} in veronal buffer, ionic strength of 0.1 at pH 8.6. The optical rotation is $-63°$. The isoelectric point is pH 5.1 ± 0.1.

Freshly prepared crystalline tetanal toxin behaves in constant solubility studies like a one component system. However, upon standing at 0°C. at pH 6.0 to 6.5, the material shows a change in solubility and the solution appears to contain more than one molecular species (34). In fact, upon standing for 10 days, at least 50 per cent of the toxin shows this change in solubility. The change is accompanied by a loss of about 75 per cent of the toxicity of the solution while the flocculating capacity remains unaltered. These

results suggested that the changes observed on standing might be caused by the association of toxin molecules. This hypothesis was substantiated by the results of ultracentrifugal studies (26, 34). The crystalline toxin described above which had stood for 10 days at 0°C. when analyzed in the ultracentrifuge showed that 55 to 60 per cent of the molecules had a sedimentation constant of 7 and that the remaining molecules sedimented at 4.5 S. The measurement of the lethal activity revealed that the toxin is associated with the molecules sedimenting at 4.5 S. Both fractions flocculated with antitoxin. These results suggest that crystalline tetanal toxin is spontaneously converted to a dimer which flocculates but is atoxic. It was further noted that this spontaneous conversion of toxin does not occur in the presence of human albumin (4.5 S). Ultracentrifugal analysis of a material composed of equal parts of freshly prepared crystalline tetanal toxin and human albumin revealed that the mixture was homogeneous with a sedimentation constant of about 4.5. On the basis of these studies it appears that tetanal toxin has a sedimentation constant of 4.5 Svedberg units. The recognition of the possibility that toxin may easily convert to a toxoid should lead to a better evaluation of the physical and chemical data on these substances. That such a conversion might have taken place with other toxins is suggested by the absence of constant solubility data for purified toxins. It seems highly probable that the methods in general use for the fractionation of toxins which involve dialysis and extraphysiologic conditions of pH, salt concentration, and temperature may have caused some conversion of the toxin.

Crystalline tetanal toxin does not precipitate anti-*Clostridium tetani* rabbit serum (26). The final product contains between 3,400 and 3,600 Lf and about 66×10^6 mouse M.L.D. per mg. N. The toxin is almost immediately detoxified by very small amounts of formalin (0.01 to 0.001 per cent). This detoxified material is highly antigenic and can be used for immunization. However, the inherent technical hazards to personnel during the processing of the toxin makes it advisable to employ crude toxoid for the parent material when a purified toxoid is desired. Methods involving methanol as the organic precipitating agent have been in use for the purification of tetanal toxoid and diphtherial toxoid and are currently employed in industry (7, 8, 9).

The mode of action of tetanal toxin is entirely unknown.

Search for clues as to the mechanism of tetanus intoxication is hampered by the absence of any pathological lesions (35, 36). The striking muscular rigidity and intermittent periods of intense motor activity suggest that there is an underlying imbalance in some regulatory mechanism in neuromuscular physiology. The normal neuromuscular relationship might be upset by an interference with the energy yielding biochemical reactions that are vital for normal nerve and muscle metabolism.

Muntz[2] (37) has made a study of the effect of tetanal toxin on certain exergonic reactions. The effect of crystalline toxin upon the glycolysis of mouse brain was tested *in vitro*. Using brain homogenates as described by Utter, Wood & Reiner (38), it was shown that 70,000 M.L.D. of toxin had no effect on this process.

The orderly formation and breakdown of adenosinetriphosphate (ATP) is a prerequisite for normal nerve and muscle function. Respiratory processes serve to maintain the supply of ATP while enzymes associated with both nerve and muscle aid in metabolizing ATP so that the energy derived from it may do useful work. Adenosinetriphosphatase is necessary for the breakdown. To study the effect of tetanal toxin upon adenosinetriphosphatase, mice were injected with 7,000 M.L.D. intraperitoneally. After five hours, these mice began to show symptoms which are typical for the acute syndrome following massive doses of toxin (35) and samples of the cervical spinal cord, medulla and leg muscle were then removed and analyzed for their adenosinetriphosphatase activity (39). No difference in the adenosinetriphosphatase activity of tissues from normal controls as compared to toxin injected animals was observed.

Lethal doses of cyanide, azide, or fluoride, substances which interfere with energy yielding metabolic reactions, give rise to a characteristic pattern of chemical changes in the blood of rabbits (40). Blood glucose may rise to four times the normal value within four hours and lactic acid may increase from eight to ten times the normal value. Muntz observed no such changes in mice which were injected with 17,000 to 35,000 M.L.D. Neither glucose nor lactic acid changed appreciably or consistently in toxin-treated as compared to normal animals.

[2] These experiments were carried out as a collaborative study between J. A. Muntz of the Department of Biochemistry, School of Medicine, Western Reserve University and one of the authors, Louis Pillemer.

An indicator of over-all energy metabolism is the metabolic rate. This was determined on normal and tetanized animals by measuring their oxygen consumption and calculating from this the calories produced per square meter of body surface per hour. Muntz found that his normal rabbits had a metabolic rate of 29.4 Cal. Following the injection of 50,000 mouse M.L.D.'s of crystalline toxin into the ear vein, the metabolic rate of those animals rose steadily until after 27 hours, when the animals had become completely paralyzed, the metabolic rate averaged 46.5 Cal. Rats behaved in a similar manner.

All of this work suggests that there is no interference with energy metabolism in tetanized animals; on the contrary, they appear to have an accentuated metabolism. This is probably a response to the generalized tetanus and increased motor activity.

Acetylcholine plays an important role in the maintenance of the normal nerve-muscle relationship (41). One of the important regulators of acetylcholine concentration in nerve fibers and at the neuromuscular junction is cholinesterase. It has been reported that tetanus toxin exerts its effect by inhibiting cholinesterase (42). Muntz investigated the cholinesterase activity of nerve tissue from normal and tetanized rabbits by the method of Nachmansohn & Rothenberg (43). The gray matter from the lumbar cord as well as samples of medulla and caudate nucleus were analyzed. The tetanized rabbits were killed 30 hours after receiving 100,000 mouse M.L.D.'s of tetanal toxin in the ear vein. Cholinesterase activity in the nerve tissue of these animals did not differ from that of normal animals.

The idea that tetanal toxin might act as an enzyme in nerve tissue to cause the liberation of free amino acids or ammonia was investigated. Mice were injected with 100,000 M.L.D. of tetanal toxin intracerebrally and after symptoms developed (3 to 4 hrs.) the brains were analyzed for their total free amino acid content (44) as well as ammonia (45). The amino acid content was normal (43 mg. per 100 gm. fresh tissue) and the ammonia content did not vary significantly from that observed in normal animals.

Since the early work of Courmont & Doyon (46) it has been repeatedly suggested that tetanal toxin acts as an enzyme in the animal body to liberate a strychnine-like substance which is really the substance responsible for the observed symptoms. Muntz conducted many experiments wherein nerve and muscle

tissue, together and singly, were incubated with massive doses of tetanal toxin *in vitro*. Alcohol extracts were prepared from such mixtures and assayed for their toxicity in mice by intraperitoneal or intracerebral injections after the alcohol had been removed and the residues taken up in physiological saline. Although some of these extracts proved to be toxic, the symptoms were not those elicited by tetanal toxin but resembled a shock syndrome. Water and saline extracts proved to be more toxic than alcohol extracts, and since control experiments, wherein no toxin was added, also often gave toxic extracts, it was concluded that the symptoms could have been due to the high concentration of various tissue extractives in such extracts. Extracts prepared from the tissues of tetanized rabbits also yielded no specific toxic substance.

Ambache, Morgan & Wright (47, 48) found that small amounts of tetanal toxin injected into the anterior chamber of the rabbit's eye results in a dilatation of the pupil and a loss of reaction to light. This reaction was specific and could be prevented by antitoxin. This effect could also be produced in the sympathetically denervated iris. The authors suggest that tetanal toxin does not affect the smooth muscle or the adrenergic nerve fibers in the iris, but that it paralyzes specifically the cholinergic nerve endings of the oculomotor nerve. During the period of prolonged dilatation, they found that the acetylcholine contents of both aqueous humour and the iris was markedly diminished. The cholinesterase content of the iris or in skeletal muscle was not affected by tetanus intoxication. They suggest that tetanal toxin interferes with acetylcholine liberation at the cholinergic nerve endings within the iris.

It is generally agreed that *tetanus bacilli* do not contain tetanal toxin as a part of the cellular constitutents. This opinion is based on the fact that tetanal toxin cannot be extracted from washed organisms with water or physiological saline. Recently, Raynaud (49) has shown that a toxic substance can be extracted from washed *C. tetani* organisms by *M* sodium chloride and 0.1 *M* sodium citrate. This substance resembles tetanal toxin. The importance of this observation is obvious and, if confirmed, will lead to intensive experimentation upon the role and interactions of the toxin with cellular components of both the bacterium and the host.

Botulinum Toxin

The isolation and crystallization of botulinum toxin, Type A, was reported independently by Lamanna, McElroy & Eklund

(50, 51) and Abrams, Kegeles & Hottle (15). The former investigators isolated the toxin from cultures grown on media containing casein, glucose, and alkali-treated cornsteep liquor. The toxin was precipitated from the culture at pH 3.5 and dissolved in a sodium acetate-sodium chloride solution at pH 6.5. The solution was shaken with chloroform under carbon dioxide and the aqueous supernatant was fractionated with ammonium sulfate. The toxin was redissolved in the pH 6.5 sodium acetate-sodium chloride solution and reprecipitated at pH 5.0. The toxin was further fractionated with ammonium sulfate at controlled hydrogen ion concentrations. It was crystallized from buffered ammonium sulfate in the pH range of 5.5 to 7.1. It can be recrystallized by redissolving the crystals in distilled water at pH 7.5 to 8.5, at room temperature, and placing the solutions in a refrigerator. In another method, the crystals were suspended in a solution of sodium chloride and sodium acetate at pH 4.0 and the suspension was dialyzed against this solvent in the refrigerator. The toxin was salted out with ammonium sulfate and recrystallized from distilled water at pH 7.5 to 8.5. The crystals appear to be needle-shaped plates averaging about 85 μ in length and 5 μ in width. The purified toxin contains 220 to 240×10^6 mouse LD_{50} per mg. N. This preparation contained 14.3 per cent nitrogen. Buehler, Schantz & Lamanna (33) found 16.29 per cent nitrogen, 0.052 to 0.059 per cent phosphorus, and 0.437 per cent sulfur. The nitrogen value of 16.29 per cent appears to be more accurate since it was determined on a sample dried to constant weight at 100°C. under high vacuum.

Abrams, Kegeles & Hottle (15) isolated the toxin from cultures grown on media similar to that used by Lamanna, McElroy & Eklund, except that a tryptic digest of casein was used instead of whole casein. The toxin was precipitated from the culture at pH 3.5, dissolved in sodium acetate at pH 6.5, reprecipitated and redissolved under the same conditions. Fractionation with ethanol at 4°C. at pH 6.5 followed by fractional precipitation with sodium sulfate resulted in a toxin solution containing 100×10^6 mouse M.L.D. per mg. N. These authors have modified the original method, which gave low yields, by using buffered phosphate instead of acetate as the solvent, by eliminating the alcohol step, and by changing the conditions during the sodium sulfate fractionations. The final toxin solution contained 500×10^6 mouse M.L.D. per ml. and 220×10^6 mouse M.L.D. per mg. N. The final yield was 34 per cent which apparently was higher than that obtained

by Lamanna, McElroy & Eklund. The crystallization of the toxin was accomplished by dialysis against ammonium sulfate at pH 6.8 and recrystallization at this pH from a phosphate buffer by dialysis against ammonium sulfate. The crystalline toxin contained 220×10^6 mouse M.L.D. per mg. N, 110,000 M.L.D. per Lf, and 2,100 Lf per mg. N. It was found to be a heat-coagulable protein, with the properties of a globulin, containing 14.1 per cent nitrogen and 0.1 per cent phosphorus. The isoelectric point was estimated to be pH 5.6 from electrophoretic mobilities.

The crystalline toxin prepared by Lamanna, McElroy & Eklund was found to be electrophoretically homogeneous at pH 4.38 in 0.1 *M* sodium acetate buffer with a mobility of 2.75×10^{-5} (52). Using the Lamanna, McElroy & Eklund method, but omitting shaking with chloroform, the mobility was found to be 2.69×10^{-5} (53). Both preparations showed a relatively low degree of reversible boundary spread. Crystalline toxin prepared by the method of Abrams, Kegeles & Hottle was found to be electrophoretically homogeneous in acetate buffer, ionic strength of 0.06 at pH 4.12 with an approximate mobility of 4.1×10^{-5} (15).

Putnam, Lamanna & Sharp (52, 53) and Kegeles (54) have studied the various preparations of crystalline botulinum toxin using a number of physical methods. The diffusion constant, D_{20}, for the Lamanna, McElroy & Eklund preparation was found by Kegeles to be 1.79×10^{-7} cm.^{2} sec.$^{-1}$ and by Putnam, Lamanna & Sharp to be 1.87×10^{-7} cm.^{2} sec.$^{-1}$ by two different methods, whereas the preparation of Abrams, Kegeles & Hottle was found by Kegeles to give a mean value of 2.13×10^{-7} cm.^{2} sec.$^{-1}$. The sedimentation diagram of the Lamanna, McElroy & Eklund preparation showed a single sharp sedimentation boundary and a sedimentation constant, S_{20}, of 17.3. There appeared to be increased boundary spreading which may have been due to molecular heterogeneity. The apparent molecular frictional ratio, f/f_0, was calculated for the Lamanna, McElroy & Eklund preparation to be 1.76 using sedimentation and diffusion data and 1.45 using viscosity measurements for the Lamanna, McElroy & Eklund and Abrams, Kegeles & Hottle preparations. Kegeles, using density measurements, determined the partial specific volume constant, V_{20}, to be 0.755 for the Abrams, Kegeles & Hottle preparation. The partial specific volume constant calculated from complete amino acid data was found to be 0.736 (53). On the assumption

that the molecules resemble prolate ellipsoids, $f/f_0 = 1.76$ corresponds to a ratio of major to minor axis (b/a) of 14.6, hydration being neglected. Putnam, Lamanna & Sharp (52, 53) calculated the molecular weight to be 900,000 from the sedimentation, diffusion and partial specific volume constants, whereas Kegeles (54) using the Abrams, Kegeles & Hottle preparation calculated the molecular weight to be 1,130,000 from the diffusion, frictional ratio and partial specific volume constants assuming an elongated ellipsoidal molecule. From the data for cysteine and cystine (33) a minimum molecular weight of approximately 45,000 was calculated, which is 1/20 of the molecular weight of 900,000 calculated by physical means. A molecular weight of 900,000 suggests the presence of 2.1×10^7 molecules per LD_{50} (52). Preliminary solubility studies have indicated more than one component (53).

Buehler, Schantz & Lamanna (33) found 19 amino acids in the crystalline botulinum toxin which accounted for all of the molecule. Aspartic acid, tyrosine, and threonine are present in greater amounts than generally found in proteins, including tetanal toxin (tyrosine was not determined) (32). The percentage of aspartic acid (calculated as asparagine) is greater than that of glutamic acid and the percentage of isoleucine exceeds that of leucine. These relationships are similar to that found for tetanal toxin. The minimum molecular weight of botulinum toxin was estimated to be approximately 45,000.

The crystalline toxin of Lamanna, McElroy & Eklund was found to be antigenic and to behave serologically as a single component (55). Both toxin and antitoxin are completely precipitated in the equivalence zone. One unit of horse antitoxin neutralizes 3×10^{-4} mg. N of crystalline toxin. Hottle & Abrams (56) found approximately 1,400 Lf per mg. N for one preparation and 2,100 Lf per mg. N for another preparation (15). The Lf was not found to be a reliable measure of antigenicity, particularly with the botulinum toxoid (55, 56). In the quantitative precipitin reaction, there exists a curvilinear relationship between the ratio of antibody to antigen in the precipitates and the quantity of antigen used (55). This toxin differs in precipitin behavior from ordinary protein antigens which usually give a linear relationship between the antibody and antigen ratio and the amount of antigen added. No serological differences exist between toxin prepared by shaking in the presence or absence of chloroform. The toxin has the interest-

ing property of forming insoluble complexes with normal sera. Lamanna (57) has recently reported that botulinum toxin causes agglutination of chicken, guinea pig, rabbit, sheep, and human red cells. This hemagglutination is apparently not accompanied by evidence of adsorption of the toxin on the red cells and it is specifically prevented by Type A antitoxin.

Crystalline botulinum toxin, Type A, does not satisfy all of the criteria for purity of proteins. The preliminary experiments using constant solubility have indicated the possibility of more than one component. The physical and chemical characterization of the toxin was not carried out on the same or similarly prepared samples and the composite physical and chemical data were accumulated on a variety of preparations. It would be of great value to have a complete analysis carried out on both the Lamanna, McElroy & Eklund, and Abrams, Kegeles & Hottle crystalline toxins in order to be able to compare them. In the calculation of molecular weight, it was assumed that the molecule was an elongated ellipsoid but electron microphotographs of inactive formalin treated toxin (toxoid) showed particles nearly spherical in shape (51). The values of 900,000 and 1,130,000 for the molecular weight which were calculated using different physical data may be incorrect if the molecule is found to be spherical.

Botulinum toxin, Type B ("okra" strain), has been isolated and purified by Lamanna & Glassman (58) from cultures grown on media containing casein, glucose, and alkali-treated cornsteep liquor. The toxin was precipitated at pH 4.0 and the resulting precipitate was dissolved at pH 2.0. Further purification was accomplished by repetition of the first steps with a final precipitation at pH 5.0 to 5.5. This method gives a 50 per cent recovery of the toxin from the original cultures. The purified toxin appears to be a protein which is soluble in water on the acid side of the isoelectric range, pH 5.0 to 5.5, and relatively insoluble on the alkaline side and within the isolectric range. It appears to be a simple protein with a nitrogen content of 15.5 per cent. The purified toxin is homogeneous by electrophoresis in glycine buffer at pH 3.8 but shows two components in glycine buffer at pH 1.8. The diffusion constant at pH 2.0 was found to be 7.22×10^{-7} cm.^{2} sec.$^{-1}$. Assuming a spherical shape, this indicates a radius of 2.9×10^{-7} cm. and a molecular weight of about 60,000. The chemical, physical, and serological properties of the Type B toxin are

different from those of Type A toxin. Its toxicity is slightly less than that reported for the Type A toxin: 110 to 200×10^6 mouse LD_{50} per mg. N and 32×10^6 guinea pig LD_{50} per mg. N. The guinea pig was found to be three times as sensitive to this toxin as the mouse, as measured by intraperitoneal injections, on a body weight basis.

The principal action of botulinum toxin is probably at the myoneural junction, possibly in the terminal nerve fibrils. This toxin acts peripherally at nerve endings and not on the central nervous system (59).

Diphtherial Toxin

Diphtherial toxin has been isolated by Eaton (60), Pappenheimer (61), and Norlin (62), who modified Pappenheimer's method. Eaton (60) separated the toxin from a proteose-peptone medium by a series of fractionations involving ammonium sulfate at pH 7.0, aluminum ammonium sulfate at pH 6.0 and cadmium chloride at pH 6.0. The Lf yield averaged somewhat over 50 per cent. Using serological methods, small amounts (between 0.5 to 2.0 per cent) of bacterial protein could be found in the purified preparations. The toxin contained about 16.0 per cent nitrogen, less than 0.5 per cent phosphorus and no sulfur. It contained 2,000 Lf per mg. N and 50,000 M.L.D. per mg. N.

Pappenheimer (61) prepared purified diphtherial toxin from a gelatin hydrolysate medium by a series of repeated fractionations with ammonium sulfate at pH 7.0. Aluminum hydroxide cream was used at various steps to remove impurities. The final products were dialyzed against sodium bicarbonate and distilled water. The best yield obtained was approximately 30 per cent. Using serological methods, small amounts of bacterial protein could be found in the purest preparations. Approximately 98 to 99 per cent of the nontoxic bacterial protein was removed. The final product contained 16.0 per cent nitrogen, 0.75 per cent sulfur and less than 0.05 per cent phosphorus. Purified diphtherial toxoid contains no sulfur (8). The isoelectric point was found to be pH 4.1. This value is different from that obtained for purified diphtherial toxoid which is pH 4.7 (8). The toxin is extremely sensitive to hydrogen ion concentrations greater than 6.0. The toxin contained 2,170 Lf per mg. N and 30 M.L.D. per Lf. Pappenheimer & Robinson (63) estimated that the pure toxin contained 2,100

to 2,400 Lf per mg. N from a quantitative study of the flocculation reaction between diphtherial toxin and antitoxin. This method is independent of the purity of the toxin used but gives the maximum toxin nitrogen per Lf in the equivalence zone. These values compare very favorably with those obtained for the toxin in the purest fractions, namely, about 2,200 Lf per mg. N.

Peterman & Pappenheimer (64) and Pappenheimer, Lundgren & Williams (65) have studied diphtherial toxin using a variety of physical methods. The toxin migrated as a single major component in the Tiselius electrophoresis apparatus at pH 7.35, ionic strength of 0.1 with a mobility of 4.9×10^{-5}. A small amount (2 to 5 per cent) of a faster component could also be observed, probably the nontoxic bacterial protein. The toxin was found to be homogeneous in the ultracentrifuge. In a 0.5 per cent solution in various buffers containing sodium chloride, the average sedimentation constant, S_{20}, was found to be 4.6, between pH 5.6 and 10.1. The pH stability range is apparently between these limits. The diffusion constant, D_{20}, was found to be 6.0×10^{-7} cm.^{2} sec.$^{-1}$ and the partial specific volume was calculated to be 0.736. From these constants, the molecular weight was calculated to be 72,000 (66). The frictional ratio, f/f_0, was found to be 1.22 and the ratio of major to minor axis, b/a, was estimated to be 4.7.

Norlin (62) modified Pappenheimer's method using repeated fractionations with ammonium sulfate followed by adsorption of the toxin on aluminum hydroxide cream and elution with phosphate. The degree of purity obtained was approximately 2,200 Lf per mg. N which was the same as that obtained by Pappenheimer. The yield of final product was about 18 per cent.

Pappenheimer (1, 67) suggested that diphtherial toxin may be the protein portion of an iron-porphyrin-containing protein, possibly cytochrome-*b*. While Pappenheimer's hypothesis is attractive, proof that a relationship between diphtherial toxin and cytochrome-*b* exists is lacking. He noted that cytochrome-*b* is serologically distinct from diphtherial toxin. Cytochrome-*b* is also nontoxic. Therefore, as Pappenheimer points out, until it is shown that diphtherial toxin has a specific effect on cytochrome-*b* activity in the intoxicated animal, or until cytochrome-*b* can be dissociated into diphtherial toxin and hemin, or until a compound having cytochrome-*b* activity can be synthesized from hemin and diphtherial toxin, the relationship of either the mode of action or

the nature of diphtherial toxin to cytochrome-*b* remains hypothetical.

Woiwod & Linggood (68) have recently observed that diphtherial toxin purified by the method of Pope & Linggood contains tyrosine, whereas hydrolyzed diphtherial toxoid prepared by the same method is lacking in tyrosine. While the meaning of this phenomenon is obscure, these authors offer the possible explanation that the amino group of the tyrosine present in diphtherial toxin is not involved in a peptide linkage, but is free to combine with formaldehyde during the process of toxoiding.

Pestal Toxin

An investigation has recently been carried out to isolate the toxin of *Pastuerella pestis* in order to study its physical, chemical, and immunological properties as well as its mode of action (30). The pestal toxin was prepared as follows: A highly toxigenic strain of avirulent plague bacilli was grown on hormone agar at 28°C. for 72 hr., harvested in physiological saline and precipitated with two volumes of acetone at −70°C., washed in the cold several times and dried under vacuum. This powder was twice extracted with 2.5 per cent sodium chloride solution (pH 7.0) at 4°C., centrifuged, and the supernatant was used for the fractionation studies.

A purification procedure involving five steps was developed after extensive trials in which methanol concentration, pH, and ionic strength were varied. The toxin was precipitated from the sodium chloride extract at pH 5.5 in 40 per cent methanol, ionic strength of 0.23 at −5°C. Impurities were removed in the next step as a precipitate at pH 5.0, ionic strength of 0.04 at 0°C. The toxin was reprecipitated at pH 4.7 in 40 per cent methanol, ionic strength of 0.04 at −5°C. In the fourth step, more impurities were removed at pH 4.9, ionic strength of 0.04 at 0°C. The final precipitation was carried out at pH 4.0, ionic strength of 0.21 at 0°C. Thirty per cent of the nitrogen was removed from the final product by dialysis against distilled water.

The final product in a 0.5 per cent protein solution has 70,000 mouse LD_{50} per ml. and 86,000 mouse LD_{50} per mg. N (or on the basis of dry weight 1 LD_{50} represents 0.09 μg.). This is, indeed, the most lethal toxin which has so far been obtained from gram negative bacteria. It should be mentioned that the biological assay is subject to great error so that the above values cannot be considered

absolute. Chemical analyses showed the toxin to contain 13.4 per cent nitrogen, 19.0 per cent carbohydrate, 2.5 per cent phosphorus, and 1.5 per cent sulfur. Electrophoretic studies indicate more than one component.

Staphylococcal Toxin

The solubility of staphylococcal toxin in methanol-water mixtures of controlled pH, ionic strength, and temperature has been determined (25, 31). The toxin is precipitated from the parent filtrate at pH 4.0 in 15 per cent methanol, ionic strength of 0.11 at −5° C. It is reprecipitated at pH 4.3 ionic strength of 0.07 at 0°C., and the toxin is extracted from this precipitate with 0.15 *M* sodium acetate buffer, pH 5.0. Impurities can be removed as a precipitate in the next step at pH 6.2 in 10 per cent methanol, ionic strength of 0.13 at −5°C. The preparation has over 2,000 Lf per mg. N. Dermonecrotic, lethal, and hemolytic activity are associated with this purified alpha toxin. Quantitative immunochemical studies (31) on the staphylococcal toxin-antitoxin flocculation reaction have indicated that staphylococcal toxin contains between 2,200 to 2,500 Lf per mg. N. Therefore, the toxin described above has been prepared in a highly pure state.

Pertussal Toxin

Pertussal toxin has been separated from a variant strain of *Hemophilus pertussis* grown in peptone broth (69, 70). The toxin can be extracted from lyophilized organisms with 0.05 *M* calcium chloride (28) and is quantitatively precipitated from this solution at pH 4.4 in 15 per cent methanol at −5°C. (29). It can be reprecipitated at pH 5.3, ionic strength of 0.15 at 0°C. The partially purified toxin contains about 10,000 mouse LD_{50} per mg. N and shows at least two components, electrophoretically, in veronal buffer, ionic strength of 0.1 at pH 8.56.

Function and Mode of Action of Toxins

The availability of pure toxins should intensify efforts to determine the function and mode of action of toxins. However, such studies are confronted with numerous difficulties and appear to depend more on novel and original ideas and new techniques than on the exploitation of existing methods. Paradoxically, the toxin, in killing the host, endangers the survival of the organism producing

the toxin. For this reason two aspects have to be considered in order to elucidate the function fully, i.e., the function of the toxin in the organism producing it, and the properties that render it toxic to the host. A theory bridging these two aspects was formulated by Papperheimer (67) in the case of diphtherial toxin, in which he suggested that a respiratory enzyme of the bacterium may act as an inhibiting analogue in the host. That such a connection may exist is an attractive idea, but it cannot be assumed a priori that this must be the case with every bacterial toxin. Therefore, two functions must always be elucidated; one relating to bacterial, the other to mammalian metabolism. The function in the bacterial cell may be concerned with digestive and respiratory enzyme systems. It is, of course, also possible that the toxin enters into the actual structure of the bacterium and has no other function. The nature of the action of the toxin on the host may not be significantly different from that of the action of pharmacologic toxins, i.e., it may block vital systems; it may act as an analogue of a slowly released physiological trigger substance in some biochemical system; it may push some equilibrium reaction entirely in one direction; or it may be an enzyme which produces a toxic substance from substrates normally present in tissues.

The physiological approach to the problem is indirect and helps only to initiate classical biochemical procedures, which involve isolated enzyme systems and the influence of the toxin on the equilibrium set up by the enzyme system. The biochemical approach is limited by the fact that the number of enzyme systems to be investigated is so enormous that the chances of finding the correct system by a method of trial and error is exceedingly small.

The problem of the action of toxins is somewhat related to that of antibiotics. However, the antibiotic is a poison for the bacteria whereas the toxin is an antigenic poison for higher animals and man. In the antibiotic field, most of the work has been centered around the concept of analogue inhibition, although Albert *et al.* (71) have presented the idea that antibiotics might act as chelating compounds which disorganize a trace metal mechanism essential for the metabolism of the bacterial cell.

There is, however, this important difference between the problem posed in studies on antibiotics and that which concerns bacterial toxins. Antibiotics are relatively simple organic compounds and their structural formulae are known or may eventually

be determined and can be used as a basis for comparison of different compounds. Bacterial toxins however seem to be proteins, and protein chemistry has not yet advanced sufficiently to give information as to structure. Furthermore, analytical chemistry can now reveal only the amounts of amino acids in a protein. Important as this is, such data are no more likely to reveal similarity between functional proteins than an attempt to identify two organic compounds by a comparison of their carbon, nitrogen, and sulfur contents. Protein function is presumably dependent upon the distribution of charges on the surfaces. Distribution of charge, in turn, depends upon the steric configuration and sequences of the amino acids.

There is another aspect which must be taken into consideration in analyzing the possible mode and site of action of toxins. This aspect has been considered at great length in the elucidation of the action of narcotics and has recently been discussed in conjunction with the action of a series of alcohols on insects (72). It has been shown that the rate at which a drug penetrates a cuticle may determine its activity. The membranes of mammalian cells differ from this model system, however, in that the products of cell metabolism must also pass through them. It is well known that this function is highly specific and selective. (The properties of the membranes of erythrocytes which have been studied extensively illustrate this point.) Any agent which would interfere with the permeability of such membranes would possibly interfere with the metabolism of the cell and the type of interference would clearly depend on the type of membrane involved. Kidney function, the chloride shift in erythrocytes, differential absorption from the intestine, and the behavior of muscle membranes point to the fact that cell membranes differ in different tissues. Therefore the specificity of bacterial toxins for particular groups of tissues could well fit into a pattern of membrane interactions. An altered permeability of the membrane could occur either by an enzymatic breakdown of selected parts of the surface which would affect permeability or by interpenetration which must also alter the permeability of membranes and their electrokinetic potential.

The biophysical approach in modern immunochemical studies which has been so important in characterizing proteins is not likely to aid greatly in elucidating function because most of the available tools furnish only constants relating to gross shape, size

and charge. There are however two techniques which may give information on the dynamic relationships of proteins: light-scattering apparatus and electron-microscopy. These may ultimately yield functional information, not so much by giving further details on the shape of individual proteins, but by giving new information on protein interactions.

All in all, this field seems to wait for the introduction of new techniques and approaches to make use of the toxins which have been isolated during the last ten years.

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NATURE AND VARIABILITY OF DISEASE RESISTANCE IN PLANTS

By Helen Hart

Division of Plant Pathology and Botany, University of Minnesota St. Paul, Minnesota

Pathogenic microorganisms and the host plants they invade are dynamic biological entities, each struggling for existence, for food and for opportunity to grow and multiply. Disease reaction in a host plant invaded by a pathogen must be judged, to great extent, by gross symptoms following attack. Resistance, an attribute of the host, is intimately related to parasitism and pathogenicity, attributes of the invader or the infectious agent. There are several stages during the process of parasitism at which a host may oppose or resist a pathogen. At the very beginning of parasitic attack, a host plant may actually exclude a pathogen or hinder its entry. Somewhat later, after a pathogen has gained entry, the host may restrict the activities of the invader. Morphological or chemical barriers may confine the pathogen to particular tissues. Physicochemical conditions of host cell membranes may limit progress of the pathogen. Lack of suitable nutrients or presence of inactivators within the host may prevent growth and multiplication of the parasite. A host plant may produce antitoxic materials to counteract the injurious effects of the invader, or occasionally the host produces materials that are toxic to and destroy the pathogen.

Interactions of hosts, pathogens, and environments are so variable that almost every case of resistance may be somewhat different from another case. In practice, resistance nearly always is relative and must be measured against a standard of susceptibility set up for a given host plant in conjunction with a given disease. Seldom can we assign responsibilities for resistance of an individual plant to one particular characteristic or property of that individual. More often the host plant is opposing a pathogen on several different fronts, so that under some conditions one type of resistance may be effective and in other cases or at other times another type of resistance may be operating.

The literature available on the nature and variability of disease resistance in plants cannot be reviewed exhaustively and perhaps

not even adequately in the present paper. A selection of representative reports indicating how disease reaction varies must suffice.

ENTRY OF A PATHOGEN

Direct penetration of the outer defenses of a plant is possible for many fungus pathogens. Growing mycelium or germinating spores of root rotting fungi penetrate root hairs or sometimes rootlets of plants, while leaf- and fruit-spotting fungi may gain entry directly through the epidermis of leaf or fruit. Some smut fungi enter the coleoptiles of young seedlings, other smuts infect flowering parts of the hosts. Some decay fungi penetrate directly through the outer portions of storage tissues, although many of the more common ones must enter by way of wounds. Rosenbaum & Sando (1) found young fruits of tomato very susceptible to *Macrosporium tomato*, but as fruits age and the cuticular layer thickens and is less easily punctured the fruits become more resistant. The epidermis of the barberry leaf is penetrated directly by sporidial germ tubes of *Puccinia graminis* but Melander & Craigie (2) found that species of *Berberis* vary in the ease with which the epidermis is penetrated. By measuring, with a Joly balance, the pressures required for puncture, they found that young leaves of the immune *Berberis thunbergii* are far more resistant to puncture than young leaves of the very susceptible *Berberis vulgaris*. Conidia of the brown rot fungus *Sclerotinia fructicola* may send their germ tubes directly through the cuticle of plum, cherry, nectarine, and apricot fruits. Cuticle of the yellow-cherry plum is thin in certain areas and cannot resist puncture, but a stout cuticle and underlying hypoderm in other plum varieties (3) may retard penetration of the brown-rot fungus and render infection more or less innocuous without completely excluding the pathogen. The *Sclerotinia* enters peach fruit through the hair sockets (3), and removing the pubescence by brushing (4) allows more fungus germ tubes to enter more rapidly by way of broken hairs.

Dry outer scales of yellow and red skinned onions contain water soluble chemical materials, protocatechuic acid and catechol, that protect colored onions from attack by the smudge fungus *Colletotrichum circinans* and the neck rot fungi *Botrytis allii* and *Botrytis byssoidea* (5 to 10) and from the decay fungus *Diplodia natalensis* (11). Diffusion of those chemicals into the soil water will prevent germination and growth of the fungi. The outer scales furnish ex-

cellent protection against disease attack unless they adhere loosely, slough easily, or split to expose the vulnerable fleshy scales, which occasionally happens in some of the hybrids that result from crossing white and colored varieties (12). The inner fleshy scales also have some bactericidal and fungicidal properties related to the pungency of a variety. Volatile and nonvolatile antibiotic materials occur in the fleshy scales (12). Mildly pungent varieties, with some exceptions, had higher smudge indices and more neck rot than strongly pungent varieties. *B. allii* is less sensitive to the antibiotics than some of the other onion pathogens, so that if it succeeds in passing the barrier of the outer scales it rapidly decays the succulent fleshy scales. *Aspergillus niger*, in contrast, seems to be stimulated by the materials in the outer scales but is extremely sensitive to the antibiotics of the inner scales, so that black mold usually is a superficial and mild disease.

Many pathogens enter their hosts by way of the natural openings available. Germs tubes of rust urediospores enter stomata of the cereals, the leaf-spotting *Cercospora beticola* enters stomata on foliage of sugar beet, and numerous bacterial pathogens of such crops as citrus, tobacco, soybean, and cereals enter through stomata. Lenticels sometimes afford entry for such pathogens as the potato scab fungus and the soft rot bacteria. Pool & McKay (13) found that penetration of the sugar-beet leaf by *Cercospora beticola* was effected only through open stomata and that stomatal movement was influenced by leaf maturity and by environment. Very young leaves of the beet plant, which are not susceptible to infection, have large numbers of small stomata per unit area of leaf surface, but pore length of a stoma is less than half the length of that in the stoma of a mature leaf. Air temperature between 70° and 90°F. and relative humidity above 60 per cent allow stomata on mature beet leaves to remain open throughout the day and germ tubes of *Cercospora* enter readily only if stomata are open. Hart (14) studied movement of stomata in varieties of wheat in relation to infection by stem rust and found that stomata of some varieties such as Velvet Don are slow to open in the early hours of the day and if opening is delayed until after much of the moisture has disappeared from cereal foliage the opportunities for stem rust infection are greatly reduced even though effective inoculum may be plentiful and temperatures favorable for infection. Although stomata of cereals do not open at night, their movements

during the day are influenced by environmental factors and may be expected to vary from season to season and from one planting area to another. In Canada, in 1931, Peterson (15) was unable to correlate stomatal behavior of H-44, Hope, and Pentad wheats with stem rust infection. Germ tubes of stem rust do not enter closed stomata according to Hart & Forbes (16). Leaf rust, on the other hand, produces germ tubes that do not require open stomata and Caldwell & Stone (17) found that stomatal behavior is not an important factor in the infection of wheat by *Puccinia rubigovera tritici.*

In studying infection by some bacterial pathogens, it has been observed that internal water-soaking of host tissue may enable the bacteria to gain entry. Either a natural or an artificially produced congestion of leaf mesophyll with water for a short time facilitates entry of *Bacterium tabacum* and *Bacterium angulatum* in tobacco leaves (18, 19, 20) and *Xanthmonas phaseoli* var. *sojense* in soybean leaves (21). In producing artificial epidemics of bacterial pustule on soybean Allington & Feaster (21) found that artificial water-soaking during the inoculation resulted in the best infection if done between 8 a.m. and 2 p.m. when stomata of soybean were open. As early as 1921 McLean (22) studied the structure of stomata on citrus and suggested that a broad, overarching ridge that narrows the stomatal opening in leaves of mandarin orange may interfere with entry of water and be responsible for some of the resistance of mandarin to citrus canker. The grapefruit stoma has a more accessible opening and water and the motile bacteria (*Pseudomonas citri*) enter the leaf more easily. Johnson (23) has recently studied both microscopic and macroscopic water congestion in tissues of many different plants and believes it to be an important factor in the early stage of infection by parasites. Heggestad (24) also has studied natural water-soaking and its relation to disease development in oats, corn, tomato, and tobacco. In tobaccos he finds that while expression of the character is delicately balanced with environment the tendency to water-soak may be inherent in some tobaccos while other tobaccos are relatively resistant to water-soaking. He suggests that it might be possible in breeding work to introduce a resistance to water-soaking into commercial varieties.

Soft rot bacteria sometimes invade lenticels of potato tubers, and a water-saturated soil or a high atmospheric humidity and

relatively high temperature increase the possibility of lenticel infection. Smith & Ramsey (25) state that few or no infections through lenticels will occur in the field or in transit at temperatures below 27°C. and relative humidity less than 94.8 per cent. But in moist or wet soils or with relative humidity of 98.2 per cent or higher, infection occurs at 61°F. although the invasion and decay will be more rapid at 72°F. Davidson (26) found lenticel infection in tuber initials of potatoes growing in wet soils, but again infection was retarded by soil temperatures of 18°C. or lower and was accelerated at higher temperatures. The Cobbler variety of potato growing in wet soil usually had more proliferated lenticels and the extent of proliferation was greater than in the Green Mountain variety.

The scab organism, *Actinomyces scabies,* also may enter lenticels of potato. Entry is more difficult if cells of the lenticel are small, thick-walled, so compact that few intercellular spaces occur, and quickly suberized (27, 28). Entry is relatively easy if the cells are large, more loosely arranged, and suberize slowly.

Smut fungi that penetrate the coleoptiles of cereal seedlings have a relatively short time in which to infect their hosts. Tervet (29) planted different seed lots of Anthony oats inoculated with *Ustilago levis* and found that smut infection ranged from 14 per cent in one lot to 72 per cent in another lot. The resistance of the first seed lot was correlated with rapidity of seed germination, with its corollary of a short time for infection. Whether the resistance observed should be considered as resistance or disease escape is a question, but the work illustrates the importance of growth habits and timing as they affect the variability of disease reaction.

RESTRICTION OF A PATHOGEN

Many pathogens may enter their host plants with ease, but progress within the host tissue is limited to certain kinds of tissue or is limited by barriers formed by the plant in response to the parasitic action of the invader. Passage through cell walls of White McCormick potato is more difficult for *Pythium debaryanum* than through cell walls of Bliss Triumph or Green Mountain potatoes because of more crude fiber and more secondary thickening in walls of White McCormick (30). Hyphae of *Colletotrichum lindemuthianum* penetrate cell walls of resistant bean pods more slowly than those of susceptible varieties (31).

Hyphae of rust fungi are unable to penetrate lignified cell walls of cereals (32, 33) and are restricted to tissues in which walls are of cellulose. Susceptible tissues occur in more or less isolated strands in the stem of the cereal plant, the size, shape, and degree of coalescence varying with the cereal variety, so that on some hosts the rust uredia that develop in these tissues are necessarily narrowly linear and restricted while on other hosts the uredia are broad and confluent.

Distribution of the flax wilt fungus *Fusarium lini* may be restricted in a resistant host. Schuster (34) planted the flax variety Bison in soils infested with race 6 or race 11 of *F. lini* and then determined extent of mycelium in the host by plating on nutrient agar various segments of the seedlings after they emerged. Bison was susceptible to race 6 and on the day of seedling emergence the fungus could be isolated from the primary roots although it did not reach the apex of the plant until seven days after seedling emergence. Race 11, to which Bison was resistant, was slower to invade the roots and its hyphae were restricted to root and crown tissues of Bison, never occurring in the upper plant parts. The variety Punjab, which was susceptible to both races of the wilt fungus, was readily invaded and within one or two days after seedling emergence the fungus could be isolated from all parts of the plants. Frequently stems of flax plants may produce new shoots that remain healthy and appear resistant to the pathogen. Schuster (34) never was able to isolate the fungus from such healthy parts. Gottlieb (35) observed a similar restriction of the tomato wilt *Fusarium* in Bonny Best tomato plants in which the main stem was thoroughly infested by *Fusarium bulbigenum* var. *lycopersici* and yet healthy shoots produced by the plant usually had no hyphae of the fungus.

At least a part of the resistance of Markton oats to the smut pathogen *Ustilago avenae* is due to the restriction of the hyphae within the developing tissues of the oats and the failure of the fungus to reach the growing point of its host (36).

A thick and tough epidermis may restrict the fructification of a pathogen or limit its final development and its effectiveness as secondary inoculum. A wheat variety such as Webster (33) may have such a sturdy epidermal membrane with such heavily lignified cell walls that even though the stem rust fungus develops within

susceptible tissues of the stem, most of the uredia remain subepidermal. The spores formed may be misshapen and compacted, and rupture of the epidermal layer is so difficult that many of the spores seldom are liberated.

Formation of a cork layer to wall off an invader is an extremely common occurrence. Many different pathogens may be walled off by layers of wound periderm and repetition of the process may occur if the pathogen breaks through the first barrier formed. A single example will suffice to show that varieties of a crop may differ in ability to resist disease by this means. The potato variety Menominee infected by the common scab pathogen forms cork three times as rapidly as the scab-susceptible Smooth Rural potato (37). Although *Actinomyces* attacks the resistant Menominee, it is very rapidly and effectively restricted.

Permeability of cell membranes may restrict progress of a pathogen in a plant. Using plasmolytic methods, Thatcher (38, 39, 40) studied permeability differences in healthy plants and plants parasitized by various types of pathogens. Permeability to water, urea, and dextrose was calculated. In celery tissues invaded by *Sclerotinia sclerotiorum* or by *Botrytis cinerea* the permeability increased, often in cells at some distance from the cells that were killed by the fungi. In potato hosts that were susceptible to *Phytophthora infestans* an increase in permeability was associated with the pathogenicity of the late blight fungus. Wheat varieties susceptible to a particular race of stem rust were characterized by a very local increase in permeability, while infection of the same hosts by a weakly virulent race was accompanied by a local decrease in permeability. The extremes of resistance and susceptibility, evident in the mesothetic reaction of a wheat plant to some races of rust, were associated with local decrease or increase, respectively, of permeability of the host cell membrane to solutes.

ENVIRONMENT AND VARIABILITY IN DISEASE REACTION

Environmental factors are responsible for the many physiological diseases of plants, but they also have far-reaching influences on the development of pathogens within plants. Temperature, moisture, and oxygen relations in the soil and atmosphere, soil nutrients and light probably are the factors that have been investigated most

for their effects on development of plant diseases. Obviously they may affect the pathogen directly, the host itself, or the host-parasite complex.

Much of the work on the relation of environmental factors to the susceptibility or resistance of plants to pathogens has been reviewed by Volk (41). Garrett (42) has reviewed investigations concerning soil conditions and the root-infecting fungi, while Wingard (43), Schaffnit & Volk (44), and Walker (45) have reviewed some of the work on plant nutrition and plant disease development.

Occasionally resistance of a host plant breaks down because a new strain of a pathogen appears that is more tolerant of environmental conditions that are adverse to the strains generally prevalent. Schall (46), for example, found one strain of the potato scab fungus *A. scabies* that grew reasonably well and parasitized potatoes at a soil pH of 5.0 whereas most of the previously isolated strains of the scab fungus were most pathogenic when pH was between 6.0 and 8.0. Hoppe (47) isolated a species of *Pythium* that stunted corn seedlings in soil at 20° to 24°C., a temperature usually considered too high for the seed-decay types of fungi to attack disease-free corn seed. Isolates of the potato late blight fungus, *Phytophthora infestans*, obtained by Martin (48) after outbreaks of late blight in the fall crops of Irish potato in Louisiana, were able to withstand exposure to 36°C. for six days, while other isolates from Minnesota withstood the exposure to high temperature for four days, and an isolate from New York withstood it for less than six hours.

There have been some reports of a change in virulence of a plant pathogen as a result of the continued cultivation on a moderately resistant host. Mills (49) believes that virulence of a potato strain of *Phytophthora infestans* for tomato may be built up by repeated passage of the strain through tomato plants and that such build-up is necessary for every epiphytotic of late blight on tomato in the northern United States. Seven passages were sufficient to enable the altered strain to kill tomato plants. And Armstrong & Armstrong (50) have found that many species of plants may act as resistant and symptomless carriers and harbor various species of *Fusarium* pathogenic to other plants.

Soil Temperature and Moisture

Rainfall during a growing season in Michigan was observed to influence the amounts of *Fusarium* wilt in the susceptible tomato

variety John Baer and in the resistant variety Marglobe (51). The John Baer had more wilt in seasons of heavy rainfall but there was more wilt in Marglobe in seasons of light rainfall. Both varieties were more susceptible to wilt at soil temperature of 28°C. than at 22°C. In a loam soil with moisture level at 85 per cent of saturation, John Baer was more susceptible to wilt than when the moisture level was only 60 per cent of saturation. Marglobe, however, was more susceptible to wilt at the lower moisture level. Even with changes in moisture level from 85 to 60 per cent, or vice versa, three weeks after the tomato plants had been transplanted to infested soil, the disease reaction of each variety was determined by the moisture level during the final seven weeks of the experiment.

Resistance in several species of host plants to the Armillaria root rot was greatest when soil temperatures were most favorable for root growth in the host (52). The greatest incidence of root rot occurred in citrus species and in rose at soil temperatures between 10° and 18°C. In apricot, peach, casuarina, geranium, and California pepper tree the root rot was most severe between 15° and 25°C. Bliss (52) thinks there are two critical temperatures for Armillaria root rot in any suscept, one at each extremity of the range for pathogenesis of the fungus. The lower one, which probably is below 7°C., usually lies below the minimum for root growth of the host and is not important. The upper one lies at approximately 26°C. and many of the suscepts may thrive at higher temperatures and be free of root rot infection. Smith (53) also found that alfalfa's reaction to the Rhizoctonia root canker varies according to soil temperatures, very few cankers developing before the soil warms to 20°C. in the spring or after it cools to approximately that temperature in the fall.

Air Temperature and Light

Plant viroses.—Symptoms of some of the plant viroses may be masked by high atmospheric temperatures. Johnson (54) found that potato mosaic symptoms were severe at 14° to 18°C., but that plants infected with potato mosaic rapidly produced new foliage free from symptoms at 24° to 25°C. although the virus was not destroyed when plants were exposed to 36°C. for 10 days. Tompkins (55) also found that potato mosaic symptoms are masked at 23° to 24°C. Symptoms of the sour-cherry-yellows virosis develop well at 16°C. but are masked if the temperature is 20°C. or higher (56).

Mills (57) has correlated the expression of severe symptoms of sour-cherry-yellows in eastern orchards with low temperatures prevailing after blooming. During eight years in which cherry yellows was severe the average temperature for 10 days preceding petal fall and the 10 days following it was approximately 55°F.; but for the 17 years in which cherry yellows was mild in the orchards the average temperature for the same period was approximately 61°F. The soybean mosaic virus 1 also is sensitive to temperature (58), symptoms being severe at 18.5°C. but usually masked at 29.5°C. Apparently there may be a range of temperatures over which symptom expression is favored for certain viroses. Baker & Thomas (59) report that a rose virus produces severe symptoms between 15° and 25°C. but that severity of symptoms is greatly reduced if temperatures remain below 15°C. Even storage temperatures may influence the development of leafroll net necrosis in potato tubers according to Folsom (60). Development of the virosis in the tubers is favored by 45° to 50°F. during the first two or three months after harvesting, while the incidence of the disease is very low at either 33° F. or at 70°F. Pound (61) and Pound & Walker (62) have studied the relation of mild or masked symptoms and severe or persistent symptoms to the concentration of virus in leaf tissues at low and high temperatures. When the turnip virus 1 infected horseradish the concentration of virus was low in horseradish leaves at 28°C. and the symptoms were mild or masked. At 16°C., however, there was a high concentration of virus in the horseradish leaves and symptoms were severe and persistent. Transferring infected horseradish plants from one temperature to the other resulted in changes in virus concentration.

Plant rusts.—Observations over many years have indicated that variations in temperature and light are responsible for fluctuations of rust reaction for the cereals infected by certain races or strains of rusts. Most of the changes observed were fluctuations within a reaction class (63) or were seasonal fluctuations associated with plant development and with weather (64, 65, 66). Apparently different associations of host varieties and rust races may have different ranges of environment over which uredial development occurs and a host is susceptible.

Waterhouse (67), in 1929, pointed out that changes from one reaction class to another sometimes occurred according to whether rust infected plants were grown in summer or in winter. Several

durum wheat varieties were resistant to certain races of stem rust in winter when average greenhouse temperatures were rather low and light was poor, but the same varieties were moderately susceptible or susceptible to the same physiologic races of rust in summer when temperatures were higher and light conditions were good. A similar change in rust reaction occurred in Joanette oats rusted by race 1 of the oat stem rust. Peturson (68) also reported that several varieties of oats were resistant to oat crown rust at 57°F. but lost resistance at 77°F. In 1933, Gordon (69) again reported on the change in reaction of Joanette oats to four races of stem rust. The variety was resistant to all four races at 12°C., resistant to races 1, 3, and 4 but only moderately resistant to race 5 at 16°C., but was moderately susceptible to races 1 and 5 and completely susceptible to races 3 and 4 at 24°C. At 28°C. it was completely susceptible to all four races. Newton & Johnson (70) confirmed Gordon's work on Joanette oats and also investigated the stability of stem rust reaction in other varieties of oats.

Hingorani (71), in 1947, also found variability in stem rust reaction of oat varieties with temperature and with light. An infection type 4 of race 2 on Jostrain grown at 70° to 75°F. was reduced to an X= infection type at 60°F. The X or X+ infection types produced on Jostrain at 70° to 75°F. by races 5 and 10 were reduced to infection type X− at 65°F. but were raised to type 3 or 4 at 85°F. Light intensity affected reactions of Marion oats to race 2 and of Benton oats to race 7: the varieties were susceptible to their respective races at high light intensity but resistant at low intensity. Further work, as yet unpublished, by Ibrahim (72) concerns variability of the oat variety Garry and certain selections having stem rust resistance of the Hagira type. Those varieties under ordinary growing conditions are resistant to all the prevalent races of oat stem rust. If grown at 85°F., however, prior to and after inoculation with rust, they are susceptible to race 6.

The mesothetic reaction to stem rust is especially sensitive to temperature and light as Johnson (73) found in studying rust reactions of several durum wheats to several races of wheat stem rust. Again, a rise in temperature changes the host reaction to complete susceptibility. An increase in light intensity also makes the host more susceptible.

A rise in temperature can bring about a complete change of stem rust reaction in McMurachy's Selection and five selections

of wheat from Kenya. Peterson, Johnson & Newton (74) subjected these wheats to an artificial field epidemic of stem rust in which 30 different races of rust were used as inoculum. They also tested the plants for disease reaction to individual races in the greenhouse. All were highly resistant in the field and also in the greenhouse when temperatures were moderate, but all became susceptible when greenhouse temperatures were above 80°F. Darley & Hart (75) also found a complete reversal of rust reaction in both seedlings and adult plants of Kenya wheats infected by several races of stem rust at different temperatures. At 65° and 72°F. the wheats were highly resistant, but at 85°F. they were susceptible. Johnson & Newton (76) believe that average summer temperatures for July at Winnipeg, Manitoba, at St. Paul, Minnesota, and at Manhattan, Kansas may help to account for some of the variability in stem rust reactions of the Kenya wheats at these three locations. For a nine-year period the average temperature at Winnipeg was 69.7°F., and the Kenyas have always been resistant to stem rust there. At St. Paul the average was 76.7°F., a temperature very close to the point at which a change in reaction may be expected. The Kenyas have rusted occasionally at St. Paul. In Kansas the average temperature was 84.2°F. and the Kenyas have not remained resistant at Manhattan.

Johnson & Newton (76) also found a change from resistance to susceptibility in other wheat varieties as temperature was changed from 60°F. to 83°F. Resistance of the varieties Syria, Sweden, Rhodesian, Talberg, and Eureka to races 15, 29, and 56 of stem rust broke down at the high temperature.

Still higher temperatures may be detrimental to rust, even in susceptible varieties of cereals (77). Little Club wheat is susceptible to a great majority of the known races of stem rust and infection type 4 is produced by many of the races at temperatures varying from 55° to 75° F. Little Club remains susceptible to races 56, 19, 21, and 34 if temperatures are raised to 95° to 99°F. although some chlorosis may appear with the last three races. It becomes resistant to race 49, however, at 90° to 94°F. and only the infection type 0 appears. Victory oats infected by races 3 and 24 of crown rust were even more sensitive to temperature than were the wheats infected by stem rust. At 85°F. necrotic lesions rather than normal uredia appeared.

While resistance to stem rust may be reduced or lost with a

moderate increase in temperature, the opposite is true for leaf rust and stripe rust. A reduction in temperature may result in loss of resistance. The wheat varieties Carina and Brevit infected by certain races of leaf rust, ***Puccinia triticina***, are resistant at 18° to 20°C. or higher temperatures, but at lower temperatures become completely susceptible (78, 79). Reductions in light intensity also decrease the resistance of these varieties to certain races of leaf rust. While many of the wheat varieties tested in Germany by Gassner & Straib (80) became resistant to certain races of stripe rust, ***Puccinia glumarum***, at high temperatures, each combination of host variety and stripe rust race had a particular critical temperature at which host reaction changed from susceptibility to resistance. Thus, Criewener 104 variety was highly resistant to race 9 at 21°C., but did not become resistant to races 2, 4, and 7 until the temperature was higher than 21°C. Strubes Roter Schlanstedter became resistant to race 7 at approximately 15°C., but 21° to 24°C. was needed for the expression of its resistance to races 2, 4, and 9. Heines Kolben, which was weakly susceptible to race 4 at 8° to 12°C., became highly resistant to that race at 14° to 16°C., but when this variety was infected by race 9 it remained susceptible until temperature rose beyond 24°C.

Nutrients

There have been many reports that the major fertilizer elements affect the development of plant diseases in the field. Heavy applications of nitrogen often have increased the amount of disease developing in a crop, while fertilizations with potassium or phosphorus sometimes have reduced disease incidence or have enabled plants to mature early and to escape a severe attack.

Nutrients may affect a host plant directly and they may alter its metabolism sufficiently to predispose it to disease or, on the contrary, they may make it more resistant to pathogenic attack. Occasionally nutrients may exert their effects directly on the pathogen and influence its growth and multiplication, either within the host plant or in culture on artificial media. Microorganisms have definite nutrient requirements and recent literature contains numerous reports on the utilization of various sources of carbon and nitrogen, the functioning of trace elements and growth stimulating chemicals, the oxygen relationships, and the enzyme equipment in certain plant pathogenic microorganisms. Nutrients also

may affect the host-parasite complex so that one or the other entity of the association is favored during the development of infection. Secondary factors, such as temperature, light, hydrogen-ion concentration, and water, may determine the availability of nutrients and thereby influence the expression of disease symptoms.

If nutrients affect primarily the host plant then some of the effects on the development of disease may be largely indirect (81, 82). Varying amounts of infection occur without altering the normal cellular compatibility of host and parasite. Nitrogenous fertilizers, for example, delay maturity, increase density of stand, and promote succulence in cereal crops, conditions conducive to greater numbers of rust infections and higher percentages of rust in the field (81). The obligate rust parasites always develop best in vigorous hosts. The cabbage club root fungus, *Plasmodiophora brassicae*, also develops best when the host is growing luxuriantly, when nutrients are well balanced and adequate for vigorous growth of meristematic tissue in the host (83). Other diseases, notably certain root rots, develop best in undernourished or poorly nourished plants. *Helminthosporium sativum* is most destructive to unthrifty wheat plants (84, 85) and any well balanced supply of nutrients or any general agricultural practices that favor rapid and vigorous growth of wheat may reduce the incidence of this root rot. The severity of Aphanomyces root rot of garden peas also may be reduced by early application of a complete fertilizer in the field or by increasing the total salt concentration of balanced nutrient solutions in controlled experimental work (86).

A direct effect of nutrient on the activity of a pathogen is evidenced by the relationship between the growth and virulence of *Phytomonas stewarti* and the amount of nitrogen in tracheal sap of sweet corn (87). During early stages of parasitic invasion the bacterium lives almost exclusively in the tracheal tubes and depends on materials in the transpiration stream for sustenance. Virulent strains of the bacterium have ability to use inorganic nitrogen. They will grow in tracheal sap of sweet corn if the nutrients supplied the corn seedlings are sufficient to furnish 20 to 40 p.p.m. nitrogen in the tracheal sap. They grow better, however, if nitrogen level reaches 200 p.p.m. High levels of nitrogen for muskmelon plants also result in greater invasion and greater mortality from the wilt fungus *Fusarium bulbigenum* var. *niveum*

(88). Nitrogen seems to be the important element governing growth of this *Fusarium in vitro*, and Stoddard (88) suggests that abundance of soluble nitrogen in xylem elements of muskmelon may influence parasitic invasion by the fungus. In early stages of disease the fungus is in the xylem and probably is nourished largely by materials in the transpiration stream until it invades other tissues. Gassner & Hassebrauk (89) report experiments with *Puccinia triticina* on wheat in which different nutrient solutions appear to affect directly the fungus and the expression of leaf rust. By immersing infected wheat leaves for three or four nights in potassium and phosphorus nutrient solutions the rust infection types were lowered and plants were more resistant to leaf rust, while solutions containing nitrogen, asparagin, glycocol, or urea raised the infection type and the plants became more susceptible.

The total salt concentration of balanced nutrient solutions may influence development of disease. Gallegly (90) found that increasing the salt concentration decreased the development of *Pseudomonas solanacearum* on tomato. Walker & Hooker (91) grew a *Fusarium*-susceptible strain of cabbage in a balanced Hoagland solution with salt concentrations varying from 0.5 of the standard basal solution to 3 times the standard. As salt concentration increased, development of yellows disease was retarded. A similar lag in development of *Fusarium* wilt of tomato occurred when Walker & Foster (92) increased the total salt concentration of a balanced nutrient solution supplied to a wilt-susceptible variety of tomato. Tomato wilt developed most rapidly at the lowest concentration used (0.1 of the basal solution), when both host and pathogen were growing slowly. Stoddard & Dimond (93) studied development of tomato wilt at salt concentrations weaker than those used by Walker & Foster and found that resistance of the tomato plant again increased as nutrient level was depressed below the minimal level used by Walker & Foster. Evidently there is an optimal nutrient level for development of *Fusarium oxysporum* f. *lycopersici* on tomato, and this optimum lies in the region between the minimum for normal growth of tomato and the ideal (93). The cabbage yellows *Fusarium* and the tomato wilt *Fusarium* are primarily xylem invaders and little or no cortical necrosis occurs during the early development of disease. The pea wilt *Fusarium* (*F. oxysporum* var. *pisi* race 1) is a xylem and cortex invader but

the rapidity of its development in a susceptible variety of pea is directly variable with nutrient concentration (86). The *Verticillium* wilt of tomato develops better as concentration of nutrient solution is increased (94). *Corynebacterium michiganense*, the cause of bacterial canker of tomato, is primarily a pathogen of the phloem during early stages of disease when tomato leaflets wilt; later the bacteria attack the stem cortex and a canker phase of the disease develops (95). The wilt phase of the disease in Bonny Best tomato plants increased as salt concentration of nutrient increased up to 2 times the standard. The later canker phase, however, affected more of the tall and spindly plants at the 0.1 nutrient concentration and relatively few of the short and stocky plants at the 3 times standard concentration. The canker bacterium needs an organic nitrogen, the nitrate and ammonium forms probably being inadequate (95). Peptides and amino acids probably are more readily available in the phloem which the bacterium first attacks, but at low nutrient levels the host might appropriate the restricted amounts available and grow at the expense of the parasite so that disease expression would be suppressed.

Lack of balance in nutrient solutions alters reaction to some diseases. Low content or lack of potassium accelerates the development of cabbage yellows (91) and *Fusarium* wilt of tomato (92) in susceptible varieties. It results in a severe attack of sweet corn seedlings by *Phytomonas stewarti* (96) and in greater susceptibility of tobacco to the downy mildew fungus *Peronospora tabacina* (97). Clubroot of cabbage is markedly reduced by omitting potassium in the nutrients (98). Providing small quantities of potassium (10 to 20 mg.) each day to tobacco plants made them highly susceptible to yellow mosaic, whereas providing moderate quantities (20 to 150 mg.) each day decreased the number of yellow mosaic lesions and reduced susceptibility of the plants (99). Growth of the tobacco was not retarded until potassium additions exceeded 200 mg. a day. Excesses of nitrogen accelerate development of cabbage yellows (91) and *Fusarium* wilt of tomato (92) in susceptible varieties, but retard development of bacterial wilt of tomato during the summer when days are long (90). Severe and early damping-off of conifers occurs with excess nitrogen (100). Raising nitrogen level from 20 to 100 p.p.m. increases severity of *Fusarium* wilt of cotton (101); an increase from 21 to 630 p.p.m. results in greater amount of infection by *Septoria lycopersici* on tomato (102); and an

increase from 6 to 60 p.p.m. increases the percentage of infection and hastens the appearance of symptoms of the yellow dwarf virus disease in onions (103). Sweet corn seedlings receiving a high level of nitrogen may be wilted so severely by *P. stewarti* that many of the seedlings die within two weeks (96), while nitrogen-deficient seedlings have only small necrotic lesions and little or no wilting when attacked by these bacteria.

The effects of nutrients may be modified by other factors. If temperature is maintained at 28°C., nutrients affect development of cabbage yellows in a host having an intermediate type of resistance, but at 19°C. the nutrient effect is not evident (91). Day length may bring about a change in the optimum nutrient level for development of tomato bacterial wilt caused by *Pseudomonas solanacearum*. In summer, when an 18-hr. day prevails, wilt development is greatest at 0.1 nutrient concentration; in early spring and late autumn, when day length is approximately 12 hr., wilt development is greatest at 0.5 and 1 times nutrient concentrations (90).

Apparently the form in which an element is available may influence development of disease in plants. Thatcher wheat and race 56 of stem rust seem to be in delicate balance at 60° to 75°F. and nitrogenous fertilizers may upset that balance and alter the usual X infection type produced by the rust race (104). If 400 p.p.m. nitrogen is supplied in a nitrate form, Thatcher becomes more susceptible and rust infection type may increase to X++; but if an equivalent amount of nitrogen is supplied in the form of the ammonium ion, Thatcher becomes very resistant and rust infection type decreases to 0; or 1+. This sensitivity to nitrogen type encountered with the mesothetic reaction to rust was not evident at high temperatures, nor was it evident at either low or high temperatures when the host reaction was complete susceptibility or high resistance. Mindum was highly resistant to race 56 regardless of the kind of nitrogen available to the plants (104). Resistance of cotton to *Fusarium vasinfectum* also varies with different nitrogenous compounds (101, 105). There is greater wilt severity with high levels of ammonia nitrogen than with high levels of nitrate nitrogen, especially when pH level of the nutrient solution is at 8 rather than at 6 or 4 (101). Albert (105), who compared wilt development and plant mortality when nitrogen was derived from calcium nitrate, ammonium nitrate, or ammonium

sulphate, found that two mutually dependent factors, a pH near neutrality and nitrogen derived from calcium nitrate, increased wilt resistance and decreased disease injury and mortality.

In 1917, Mains (106) suggested that the cereal rusts probably require some transitory or nascent organic products related to the carbohydrates they obtain in the living host. Mains was able to supply excised corn leaves with various sugars to support development of rust. Johnson & Johnson (107) determined sugar content of immature and fully developed tissues of wheat varieties that varied in adult plant reaction to stem rust. Although the young susceptible tissues had the higher sugar content, particularly the reducing sugars, the differences were of the same order in all varieties tested irrespective of their reaction to stem rust. More recently Gassner & Hassebrauk (108) suggested that nitrogenous compounds may be the critical materials for development of cereal rusts. Gassner & Franke (109, 110) continued the work reported by Gassner & Hassebrauk (108) and determined total nitrogen content, the protein nitrogen fraction, soluble nitrogen fraction, amide nitrogen, amino acid nitrogen, and residual nitrogen in young wheat plants. In wheat and rye varieties that usually become more resistant to *Puccinia glumarum* and to *Puccinia triticina* as temperatures rise, they found (109) that total nitrogen content was higher at 10°C. than at 20°C. and that values for the protein nitrogen were higher at the lower temperature. The proportion of soluble nitrogen, however, decreased at the lower temperature. They also determined the nitrogen fractions as they were affected by variously balanced nutrient solutions supplied to three varieties of wheat that differed in their reactions to race 14 of *P. triticina* (110). Potassium balance varied from 1/20 of standard, through standard, to 5 times standard. Nitrogen and phosphorus were absent, normal, or 5 times the normal standard. Total nitrogen values and values for all nitrogen fractions in the tissues generally increased as potassium was increased in the nutrient. Rust reactions of the very susceptible varieties and the very resistant variety did not change appreciably with those changes in host physiology. The moderately resistant variety, however, bore a 4 infection type of leaf rust at the n/20 level of potassium, a 3 infection type at the normal level of potassium, and a 2 infection type at the 5 times standard level, so that resistance was increased as more potassium was supplied. As nitrogen was increased in the nutrient,

nitrogen content of the plants rose markedly, but the increase was in soluble nitrogen rather than in protein nitrogen during the rather short experimental period of 13 days. Again, the moderately resistant variety was the one in which rust reaction changed: with no nitrogen in the nutrient the infection type was between 2 and 3, with normal nitrogen a 3 infection type appeared, and with 5 times normal nitrogen the infection type approached type 4. The effects of phosphorus in the nutrient were relatively weak and did not greatly influence nitrogen content of the plants. Because the potassium and nitrogen in the nutrients seemed antagonistic in their effects, certain nutrients containing both elements were supplied the plants. Potassium partially counteracted the effects of nitrogen, and the effect of sodium was similar to but weaker than that of potassium. An ammonium nitrate as nutrient increased nitrogen content of wheat leaves more readily than did nitrogen in the form of sodium nitrate. In all of these experiments the quantitative changes in nitrogenous fractions seemed to be concerned with changes in the reaction of the moderately resistant variety to a particular race of leaf rust, but the quantitative changes could not be held to account for the specificity of reaction to various other races of rust. When Gassner & Franke (110) determined nitrogen content for the 11 wheat varieties that are differential hosts for *Puccinia glumarum* there was no evidence that the highly specific reaction of a variety was related to nitrogen content of the tissues. They postulate that qualitative changes in specific protein units may be more important than quantitative changes.

Nitrogen compounds in potato foliage and tubers have been thought to play a role in determining resistance to *Phytophthora infestans*. Alten & Orth (111) analyzed foliage and tubers produced by potato plants supplied with varying amounts of potassium or varying amounts of both nitrogen and potassium. Tubers from plots supplied with nitrogen and phosphorus but without potassium were most susceptible to fungus attack and they also had the highest values for total nitrogen content, protein nitrogen, nonprotein nitrogen, and especially the α-amino acid in the nonprotein fraction. When both nitrogen and potassium were varied in the nutrient the content of total nitrogen in the tuber was highly dependent on the potassium:nitrogen ratio. If nitrogen was given in limited amount (100 mg. per l. of water), the total nitrogen

content of the tissue decreased as the amount of potassium in the nutrient was raised. If 200 mg. nitrogen was in the nutrient then an increase in potassium had no effect, and total nitrogen content of the tuber remained fairly constant. Increasing nitrogen to 400 or 800 mg. led to increasing values of total nitrogen in the tuber as potassium in the nutrient increased. The amino acid arginine hindered or inhibited germination of sporangia of the fungus, 0.1 per cent being toxic to the *Phytophthora*. The arginine content of potato foliage was relatively high and may have afforded some protection against foliage infection. Foliage of plants in the nitrogen-phosphorus series, without potassium, always had the lowest values for arginine, and as a rule arginine increased as potassium in the nutrient increased. In mature tubers, arginine was lowest for the susceptible varieties, but other varieties seemed not to be dependent on arginine for their blight resistance; and the authors concluded that some unknown factor in addition to arginine content was involved. Hagenguth & Griesinger (112) also analyzed potato tubers for nitrogen fractions and believed that residual nitrogen was the fraction utilized by the *Phytophthora* but their study of the very heterogeneous progeny from a selfed selection failed to give them a correlation coefficient that would indicate a definite relationship between tuber susceptibility and residual nitrogen content.

ACQUISITION OF RESISTANCE OR TOLERANCE

Some animals acquire an immunity to disease by producing antibodies that confer a specific protection against a pathogen and its toxins. The protection may be conferred for a limited time or for the life of the individual, and transfer of antibodies to a healthy individual often will prevent infection by the specific pathogen or by closely related strains. The invaded individual that produced antibodies recovers from the disease and regains its healthy state, destroys or eliminates the pathogen, and retains the protective substances that ward off future attacks. Evidence and proof for the production of antibodies by plants is extremely difficult to secure, probably because of inherent differences in animals and plants and especially because of the lack of a circulatory system in plants.

Some workers have thought that plants acquire an immunity from certain virus infections because of a partial or an apparently

complete recovery from the gross symptoms of disease and a consequent protection against a superinfection by the same or a closely related virus. Other workers prefer to term such a phenomenon acquired tolerance, because the infectious agent usually remains in the invaded tissues and does not lose its virulence for other individuals. Some of the European workers (113, 114) use the term premunity. Still other workers believe that the recovery merely constitutes a change from an acute stage of disease to a chronic stage.

Necrotic symptom patterns produced by the ring-spot virus on tobacco leaves inoculated by mechanical transfer of infectious juices fail to appear on the young foliage produced subsequent to inoculation (115 to 119). Plants appear to recover from the systemic virosis, but juices of the recovered portions are still infectious for healthy plants (115, 116, 117). If cuttings are propagated from the recovered portions the symptoms remain masked (116). Tissues that have recovered cannot be reinfected by ring-spot virus (115); they seem to have acquired protection against ordinary ring-spot virus and also the closely related green form (120). Protection is specific, however, because the tissues may be infected (120) by viruses of tobacco mosaic, cucumber mosaic, potato vein banding, potato ring spot, tomato spotted wilt, tomato etch and severe etch, and even by tobacco ring-spot virus 2. When concentration of ring-spot virus was assayed according to the necrotic primary lesions produced on leaves of Black cowpea, in recovered portions of plants it was only 10 to 20 per cent of the concentration in patterned leaves (121). Price (122) suggested that plants might acquire an immunity of the carrier type involving persistence of the disease agent but disappearance of obvious manifestations of disease.

Other evidence for an acquired immunity of the nonsterile type in plants has been presented. Tobacco plants infected by attenuated strains of the necrotising aucuba mosaic virus become immune to virulent strains, and plants infected by tobacco-mosaic virus become immune to aucuba mosaic within two days (123). Leaves that are partially mottled or affected with vein-clearing symptoms by systemic mosaic infection become immune to aucuba mosaic more slowly and only the leaf areas affected by tobacco-mosaic virus are protected against aucuba mosaic virus. Kunkel (123) does not attempt to explain the mechanism of protection

which seems to be closely associated with spread and multiplication of the virus of tobacco mosaic but he mentions the belief of Thung that only one of two closely related viruses usually occupy a given cell, the possibility of inadequate materials needed by viruses for their multiplication, and the possible production of immunizing substances by infected cells.

An acquired immunity or tolerance is characteristic of tobacco streak, another virosis in which distinct necrotic symptoms appear on inoculated leaves of tobacco. The subsequently developed leaves, however, are symptomless (124, 125, 126) and recovery of the plant seems to be rapid and complete. Concentration of virus, assayed for comparable unit areas of infected tissue, is as high or higher in symptomless tolerant leaves as in necrotic leaves, and virulence is not reduced in tolerant leaves (126). Addition of reducing agents at the time tissue was ground for assay work was necessary to prevent the effects of a virus inactivator that occurred in both healthy and diseased tobacco. Since concentration of tobacco streak virus does not vary as does concentration of ring-spot virus (121), Fulton (126) also determined ring-spot virus concentration in necrotic and in recovered tissues of two varieties of tobacco. He found approximately the same concentrations in two tissues of Havana 38 tobacco but high concentration in necrotic leaves and low concentration in tolerant leaves of the Turkish variety Xanthia.

Tomato plants may partially recover from a severe attack of curly top by regenerating relatively healthy shoots from the leaf axils (127). Slight yellowing and a purple tinge are mild symptoms of curly top in regenerated parts and occasionally a relapse occurs and the recovering plant dies. A high proportion of the population recovers in some wild strains of tomato, a low proportion in other wild strains and most commercial varieties. Turkish tobacco also may partially recover from curly top by producing very mildly affected shoots from axillary buds or by a renewal of terminal growth with progressively milder symptoms of disease (128). Clones established from recovered tomato or tobacco plants may be fairly productive in spite of their slow growth and the persistence of mild symptoms of curly top. They retain the apparent resistance or tolerance to the virus and severe symptoms do not develop if they are reinoculated with the virus. The virus, unchanged in virulence, is still present in the tissues and can be transmitted to

healthy plants by the beet leafhopper. Recovery from attack by one strain of curly-top virus protected tobacco against attack by a second strain.

Valleau (118) was unwilling to consider the loss of necrotic symptoms of ring-spot in tobacco a true recovery and set forth the hypothesis that inoculation of patternless leaves on an infected plant is without effect because the cells are already parasitized to the limit. He suggested (129) that while death of cells in mature tissue due to their hypersensitivity to the virus might result from the sudden change of a considerable quantity of precursor material into virus, the cells that are invaded while in an embryonic state may be able to make a normal adjustment so that there is only a gradual conversion of precursor material to virus. The apparent recovery could be modified by temperature (129); leaf-edge chlorosis and necrosis developed in the "recovered" leaves if plants were grown at 20°C. rather than 26°C. Valleau postulated that the ring-spot disease merely entered a different phase as soon as embryonic tissues were invaded.

Different phases of disease are evident in other viroses—an acute stage in which disease symptoms are severe and prominent and a chronic stage in which gross symptoms are not evident or are mild, inconspicuous, and more or less noninjurious. New growth from axillary buds of potato plants infected by yellow dwarf virus often has only mild symptoms of disease while foliage of the main axis bears severe necrotic patterns of an acute stage of yellow dwarf (130). Acute and chronic phases of tobacco common mosaic and yellow mosaic occur in infected Turkish tobacco (131), the severity of symptoms paralleling closely growth phases in developing leaves. The chronic mosaic appears in leaves differentiated about the time that vein-clearing is evident in inoculated leaves and thereafter. Assays by means of local lesions on bean leaves indicate that virus activity is less in tobacco leaves with chronic mosaic than in leaves with the acute phase of mosaic (131). Natural resistance of a plant is believed to vary because of differences in ability to maintain a high level of virus synthesis or because of some condition that governs movement of the virus (131).

A plant virus may be introduced on one side of a graft and will pass to the other side. By studying the passage of curly top virus through various types of graft unions, Wallace (132, 133) believed that he found evidence for the production of protective substances

in tissues of Turkish tobacco that had partially recovered from curly top. Scions from healthy tobacco plants were grafted to stocks of tobacco that had recovered from curly top and scions from recovered tobacco plants were grafted to healthy stocks (132). In each case the virus passed through the graft union and only mild symptoms developed in the previously healthy parts. There was no evidence that the virus had been attenuated, because it could always be picked up in virulent form from the various mildly diseased parts if beet leafhoppers were allowed to feed there. Scions taken from tobacco plants 5 days after viruliferous leafhoppers had been allowed to feed, at which time no symptoms had appeared, furnished virulent virus to the stock and severe symptoms developed. Scions taken 10 and 15 days after leafhopper feeding, when symptoms of moderate severity had appeared, furnished sufficient virus to the stock so that symptoms were severe for a time and then gradually became mild. If scions were not taken until 20 days after leafhopper feeding, when symptoms were severe, the stocks developed only mild symptoms.

Following Wallace's report (132) on evidence for passive immunization of tobacco to virus of sugar-beet curly top, Price (134) studied severity of curly top symptoms in tobacco as influenced by site of inoculation or route of infection. He grafted at various distances from the terminal bud and allowed insect vectors to feed at corresponding distances, and to make results more comparable he delayed insect feeding for approximately the time required for tissue union in the grafts. Mildness or severity of symptoms depended on the success with which the virus, from either the scions or the insects, reached the growing point before the plants matured. Price did not believe that protective substances were transmitted from recovered to healthy plants by grafting. He suggested that if relatively large quantities of virus gain access to the plant near the growing point and if the virus reaches embryonic tissue quickly, before the plant has opportunity to set up a defense, the symptoms are severe.

CONCLUSIONS

The works selected for review represent a rather small part of the literature available on plant disease reactions. They are not intended as a comprehensive survey of knowledge regarding complex problems that confront any plant scientists working with

the dynamic biological associations and environmental interactions involved in plant diseases. They illustrate and emphasize what may happen when plants become diseased but they do not always explain why plants and their pathogens behave as they do.

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ORAL MICROBIOLOGY

By R. W. Harrison

The Central Administration, The University of Chicago
Chicago, Illinois

The bacterial flora of the mouth is sufficiently complex (1 to 4) and variable (1, 5) to discourage attempts at complete analysis. Most of the microorganisms which are associated with oral infection, or with systemic infections having their origin in oral lesions, have been fairly well identified; so have most if not all of the usual microbic inhabitants of normal mouths of healthy individuals.

The so-called normal flora changes with the diet (6 to 11) and with the development of infection of the teeth, the gingival and the oral and nasopharyngeal mucosae. It is affected by the composition and rate of flow of the saliva (12, 13, 14), by physical structure and condition of the teeth, by the chewing of rough or fibrous foods, which have a cleansing action on the teeth, and by other local as well as systemic factors. In addition to variables such as these there is the further complication of the transitory flora introduced sometimes in large numbers with certain foods, many of them being dead when introduced and others not growing but some of them multiplying perhaps temporarily, in the mouth. Kligler & Gies (15) reported in 1915 that they were able to culture only a small fraction of the bacteria found in smears from the mouth. Others who have attempted cultivation of the total flora have experienced similar difficulty. Bibby & Maurer (16), who estimated that they obtained in culture about one-sixth of the bacteria present in the saliva samples studied, stated that they recovered in culture six times as high a proportion as had been reported by any other investigator.

Except for differences in relative incidence, sometimes marked, there appears to be little variation in the composition of the flora of different parts of the mouth (17, 18) or in the mouths of different healthy individuals (19).

Microorganisms which gain access to the mouth at birth (20) apparently become fixed inhabitants and by the time the deciduous dentition has formed the complete normal flora has been es-

tablished. The principal changes thereafter appear to be associated with marked changes in the diet or with disease.

Most of the studies on the oral flora have been made in the course of investigations on dental caries, suppurative periodontitis, tonsillitis, and other diseases of the oral cavity. For a discussion of oral lesions of systemic infection the reader is referred to a chapter by Cornbleet in Gordon's *Dental Science and Dental Art* (21) and to Prinz & Greenbaum (22), and for the bacteriology of pulpitis, periapical infection, and focal infection to Appleton (23).

Gingivitis and Periodontal Infection

For clinical and pathological descriptions of the diseases of the tissues supporting and surrounding the teeth the reader is referred to textbooks of dentistry (24 to 30). Appleton (23) discusses the oral flora primarily associated with these diseases. Discussion here is restricted principally to more recent reports concerning the bacteriology, and predisposing or complicating factors, of the more common diseases of the gums and alveolar tissue.

Gingivitis.—Any degree of inflammation of the gum margins may be referred to as gingivitis. It may range from a mild involvement of the interdental papillae or of the buccal and lingual margins (usually in association with excessive deposition of calcified material on the necks of the teeth or other traumatizing influences) (26, 29) to an acute or chronic ulceromembranous gingivitis (Vincent's infection) (26). Gingival infection may extend into the periodontal membrane leading to a suppurative periodontitis (pyorrhea) (31, 32).

Most if not all the microorganisms which have been found in suppurative periodontitis and in Vincent's infection have been encountered in mild gingivitis, although in smaller numbers and with less regularity, and have been recovered from apparently normal gingivae (33 to 38). Davis & Moorehead (39) reported in 1928 that fusiform bacilli, spirochetes, cocci, and leptothrix threads occur in large numbers in bacterial masses growing constantly along the gingival margins. Although they are most often found in the gingival groove and on the margins of the gingivae, apparently the lodgment of these organisms in the mouth does not depend upon the presence of the teeth. Rosenthal & Gootzeit (40), for example, found fusiform bacilli in more than 10 per cent of the edentulous mouths examined by them. They found spirochetes

less often and found fusiform bacilli and spirochetes in association only twice in some 200 edentulous adults.

Although, of the various microorganisms recovered from cases, streptococci of various kinds, nonsporulating anaerobes, and spirochetes and fusiform bacilli have been most frequently suggested as the cause of gingivitis, it now seems clear that inflammatory and suppurative gingival infections do not develop in the absence of other local or systemic predisposing conditions. Further investigations are needed. Among the predisposing factors that have been suggested are deficiencies in vitamins A (41), B (42 to 46) and C (47 to 51), various general health conditions (52, 53, 54) and poor oral hygiene (41, 49, 55, 56). King, who has prepared an extensive critical review of the influence of diet on parodontal disease (41), concludes that while dietary deficiency, particularly of vitamins A, B, and C, may lead to changes in parodontal tissue and in turn to gingivitis there is no more than suggestive evidence that these deficiences are etiologically important in human parodontal disease.

Vincent's infection.—This disease is an ulcerative, necrotizing gingivo-stomatitis, characterized by formation of a pseudomembrane and development of a putrid odor. During World War I when widespread among the troops it was called "Trench Mouth." It is also referred to in the literature as ulcerative stomatitis, ulceromembranous gingivitis, necrotic gingivitis, phagedonic gingivitis, and Mundfäule (putrid mouth). It is often not easily distinguishable from other forms of gingivitis, its clinical and bacteriological diagnosis is frequently uncertain (57) and its epidemiology is undefined (58 to 61). The disease is commonly regarded as a fuso-spirochetal infection but other organisms, mostly anaerobic, are almost equally characteristic of the disease whether it is confined to the oral and pharyngeal mucosa or involves other tissues, as in tropical ulcer, putrid bronchitis, and pulmonary abscess and gangrene. Infected tissue usually contains several varieties of spirochetes (4, 34, 35, 62 to 66), several kinds of fusiform bacilli (37, 38, 62, 67, 68), spirilla (4, 34, 62), anaerobic streptococci (69), and other common bacterial flora of the mouth (62). In his excellent review of the subject, Rosebury (62) states that the organisms grown in pure culture have little or no independent pathogenicity for animals but mixtures of four pure cultures: a spirochete (*Treponema micordentium* or *T. mucosum*),

a fusiform bacillus (one of several varieties), *Vibrio viridans* and an anaerobic streptococcus, produce characteristic, transmissible infection in experimental animals.

Suppurative periodontitis.—Marshall Day & Shourie (31) have indicated that the change in age incidence of gingivitis and periodontitis suggests that hypertrophic gingivitis in children progresses into pyorrhea in adults. Most investigators have found the microbic flora of suppurative periodontitis to be much the same as the flora of gingivitis. Hemmens & Harrison (33) have noted several reports suggesting that suppurative periodontitis is an infectious disease and naming staphylococci, alone or in combination with streptococci or with fusospirochetes, and amoebae alone or in combination with bacteria as specific causative organisms. Simoda (70) listed a large number of microorganisms which have been named as etiologic agents of this disease and reported the experimental production of pyorrhea in guinea pigs with a corynebacterium which he found repeatedly in pus from suppurative periodontitis. He named the organism *Corynebacterium paradentosis*. Tunnicliff, Fink & Hammond (71) found fusiforms sufficiently predominant at the base of periodontal lesions to lead them to conclude that this organism has causal relation with suppurative periodontitis. They found spirochetes and other forms to be less persistent. Klein (35) on the other hand has pointed out that spirochetes, which he found in small numbers in normal mouths, were increased in gingivitis and were most numerous in lesions of suppurative periodontitis with deep pocket formation. Davis & Moorehead (39), using especial care to prevent contamination from the flora of the gingival margins, examined the pus in 20 alveolar abscesses and found spirochetes in 16. They found streptococci and diplococci in small numbers in all cases and fusiforms in large numbers.

Hemmens & Harrison (33) found spirochetes in all of 46 cases of suppurative periodontitis studied by them. Using both aerobic and anaerobic culture methods they found little difference in the bacterial flora of normal gingivae and suppurative lesions except that fusiform bacilli and *Bacterium melaninogenicum*, as well as spirochetes, were more numerous in material from the diseased tissue. The most frequently encountered organisms from healthy gingivae, as well as from periodontal lesions, were *Micrococcus gazogenes*, *Fusiformis nucleatus*, other fusiforms, *Bacterium melaninogenicum*, gram positive cocci, bacteroids, and nonbranching,

filamentous forms which they referred to as leptotrichia or leptotrichia-like organisms. Exudate from periodontal lesions inoculated subcutaneously into scorbutic guinea pigs produced localized absecesses. Pus from these abscesses was inoculated subcutaneously into normal guinea pigs and the infection was maintained in normal guinea pigs by serial subcutaneous inoculation of pus from the resultant lesions. There was progressive increase in virulence and decrease in complexity of the original mixed flora until after six or eight passages when a stable microbic flora became established. This consisted of spirochetes (*T. microdentium?*), fusiform bacilli, *Bacterium melaninogenicum*, *Vibrio sputigenes*, a filamentous rod and several species of anaerobic cocci. This stabilized mixed flora was strikingly similar to the mixture found by Smith (69) to be similarly infective for animals. Material from normal gingival crevices similarly inoculated into guinea pigs produced more localized, less rapidly developing lesions but the flora became similarly stabilized and when finally established did not differ from that of the periodontitis series. The authors concluded that the role of bacteria in suppurative periodontitis is that of secondary invaders which may be responsible for the suppuration.

Dental Caries

After extensive review of the literature on the incidence of dental caries Rosebury stated (72) that there is much less caries among primitive races in many parts of the world than among more civilized peoples and that primitive peoples suffer a markedly increased incidence of decay when they come into contact with and adopt some of the habits of the white man. After observing that those investigators, who have studied the changing incidence of dental caries associated with growing contacts with civilized communities, are in agreement in indicating that dental caries is a disease of the white man's culture but otherwise offer little that is consistent in explanation of the phenomenon, he points out that the change in incidence must be associated either with the absence among primitive peoples of caries producing or predisposing factors or with the loss or diminution of protective factors.

The influence of heredity, diet, and physical, chemical, and microbiological factors in the oral cavity may be studied from either or both of these points of view. There is little or no evidence that

hereditary differences are involved in the lower incidence among ancient peoples (73 to 82) or among primitive or isolated cultures (72, 83 to 93), most of the investigators having attributed the differences found to dietary factors. Rosebury has reviewed (72) the earlier literature with respect to inheritable structural defect in relation to susceptibility to dental decay. Observations on siblings (94) and identical twins (95) are suggestive of inheritable resistance but it has not yet been possible to show that dietary and other environmental factors are not primarily or entirely responsible for the effects observed. Hunt and associates (96, 97, 98), using a diet (99) which predisposes to the development of tooth decay, have developed susceptible and resistant strains of albino rats by phenotypic selection, testing of caries susceptibility in the progeny of breeding pairs and brother-sister mating. They have demonstrated marked hereditary differences in suseptibility.

Marshall (100), Bibby (101), Rosebury (72), Karshan (102), Gottlieb (103) and Neumann (104) have reviewed and assessed the effects of various local and systemic physical and chemical factors on the incidence and course of dental caries. A brief analysis of these factors and their interrelationships has been made by Blayney (105) and Rosebury (72). Jay (106), Bibby (107), and Harrison (108) have reviewed the bacteriology in relation to etiology of the disease. Hemmens, Blayney & Harrison (109) in cultures from dental plaques from both carious and noncarious areas found that the predominant microorganisms were α-hemolytic streptococci, nonhemolytic, gram positive diplococci, diphtheroids, and anaerobic fusiform bacilli. Other microorganisms, less numerous and less frequently isolated, were *Neisseria*, micrococci, an unidentified small gram positive rod, lactobacilli, a small, anaerobic, gram negative coccus, and filamentous forms, both branching (actinomyces) and unbranching (leptotrichia). The occurrence of these forms in plaques from carious and noncarious areas was not markedly different. Organisms of four groups were found in cultures on media adjusted to pH 5.0: streptococci, lactobacilli, micrococci, and monilia, the last two only infrequently. Of these forms the lactobacilli were found most frequently in plaques from carious areas, streptococci most frequently in plaques from noncarious areas. The same investigators together with Bradel (110) in 1946 reported further with respect to the flora of the dental

plaque, identifying 27 varieties of microorganisms, and stating that only the lactobacilli increased in incidence with the appearance of the initial stages of carious decalcification of the enamel. Essentially the same flora has been found in association with experimental caries in the rat, induced by feeding a coarse-particle diet (111), and with experimental caries of the hamster (112). Wakeman and associates (113) have found lactobacilli and enterococci predominant in carious lesions of the cotton rat, the enterococci being more numerous. They called attention to the strongly proteolytic action of the enterococci in the dentinal lesions. Hurst *et al.* (114, 115) isolated one of the filamentous forms from dental plaques, tentatively identified as *Actinomyces israelii*, which was highly pleomorphic in culture media, and produced with it *in vitro* lesions in hamster teeth which they reported to be identical with the lesions developing *in vivo* in the presence of the complex oral flora. Dietz (116) was able to produce plaque formation and decalcification (etching) of a tooth section *in vitro* which was exposed to saliva and fermentable carbohydrate. He described plaque formation in which filamentous forms appeared late and, he stated, probably contributed nothing to the lesion. Pincus (117), who has supported the claim for proteolytic bacterial etiology of dental caries, suggested that enzymes from gram negative bacteria digest the protein sheaths of enamel prisms, releasing sulfate which brings about solution of the enamel.

Bunting and associates (7), Kitchin & Permar (8), Becks and associates (9, 10), whose experimental results are strongly indicative of a causal relationship between *Lactobacillus acidophilus* and dental caries, report simultaneous decrease in the numbers of lactobacilli in the mouth and decreased development of caries by dietary restriction of carbohydrate. Boyd and associates (118) were unable to correlate the numbers of lactobacilli with either the extent of carious damage or the rate of caries progression. They were able to establish correlation of numbers of lactobacilli with the status of mouth hygiene thus suggesting that the relationship of this organism with caries is secondary or opportunistic. On the other hand, Strälfors (119) as well as others previously cited, has found that the number of lactobacilli closely parallels caries activity.

There has been some uncertainty as to the identity of the lactobacilli of the oral cavity. Since the discovery of *Lactobacillus acidophilus* by Moro in 1900 (120) and of *L. bifidus* by Tissier (121)

organisms of this group have been found in the mouth by numerous investigators. Several investigators, including Rettger and associates (122) have found oral lactobacilli to be different from *L. acidophilus* of intestinal origin. Kligler & Gies (15), Heineman & Ecker (123), Howe & Hatch (124), Rahe (125), Bunting and associates (126), Rosebury and associates (127), Howitt (128), Howitt & Van Meter (129), Enright, Friesell & Trescher (130), Tucker (131), Warner & Arnold (132), and Harrison & Opal (133) have found oral and intestinal lactobacilli to be indistinguishable. Harrison & Opal (133) presented evidence that intestinal lactobacilli probably have their origin in the mouth. Rosebury (134) has written a critical review of the parasitic lactobacilli in which their role in oral infection is discussed.

Classification of the lactobacilli has been difficult. Hadley and associates (126) identified four morphological groups which have been used as the base for further study by most subsequent workers. Howitt (128) and Howitt & Fleming (6) found seven fermentation groups among 77 strains of oral origin. Harrison (135) divided 101 oral strains into two groups. The larger group (75 strains) coagulated milk and reduced litmus and could be subdivided on the basis of carbohydrate fermentation into five subgroups; the other, not coagulating milk nor reducing litmus, contained four fermentation subgroups. No correlation could be found with morphological groupings. Whyte (136) studied the fermentation reactions of 100 strains of human oral origin and found considerable variation in fermentative capacity. There was no correlation of fermentation reactions and morphology. Harrison and associates (137) tested the immunological relationships among 77 strains of oral origin, using a carbohydrate extracted from the cells as the test antigen, and found evidence of four specific immunological types and a heterogeneous group. A close correspondence was found between immunological typability and ability to coagulate milk (138). Some forty of the cultures studied by Harrison were found to be unstable in immunological relationship (139) and in the majority of cases change in immunological type was accompanied by change in capacity to coagulate milk and to ferment sorbitol and mannitol. Williams & Harrison (140) were able with nontoxic cellular carbohydrate extracts to elicit skin and corneal reactions in rabbits sensitized by intradermal injection of heat-killed cultures. Williams

(141) showed later that lactobacillus immune sera contain antibodies separately reactive with two or more antigens contained in the carbohydrate extracts from cultures and demonstrated the existence of four or more antigenic components thus accounting for the distribution of types and cross reactions between types. He was able also to demonstrate specific skin response to these antigenic components in sensitized rabbits (142), and to classify cultures by agglutination in monospecific antisera (143). Coolidge & Williams (144) found that changes in fermentation reaction were accompanied by changes in vitamin and amino acid requirements, in acid production and the pH attained in culture as well as by changes in antigenicity.

Practically all the investigators who have studied the bacteriology of carious lesions or of the saliva from carious mouths have noted the continuous presence of acid producing streptococci. Hammond & Tunnicliff (145) produced carious decalcification *in vitro* with *S. viridans*. Tefft (146) has stated that *S. fecalis* and *S. lactis* are sufficiently acidogenic to be significant as decalcifying agents in dental caries. Belding & Belding (147), following a series of papers on a mucoid producing streptococcus (*S. salivarius*), claim that a high carbohydrate diet causes the development of mucoid producing strains of streptococci which are active in the production of caries. Bibby (107) takes the view that any microorganism in the oral flora capable of producing acid should be considered as a potential etiologic agent in dental caries and states that the evidence indicates that streptococci may be involved.

It is beyond the scope of this review to attempt an analysis of the literature on dental caries control. There are, however, significant implications for the bacteriology of dental caries in the literature on this subject. Excellent surveys on fluorosis and dental caries are available in two published symposia on the subject (148). Additional guides to the literature on caries control with fluorides may be found in recent papers by Dean (149), Klein (150), McClure (151), Arnold (152), Bibby (153) and Knutson (154), and on other inhibitory substances in papers by Stephan (155), and Kesel and associates (156).

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DERMATROPIC VIRUSES

By G. John Buddingh
The Department of Microbiology, Louisiana State University School of Medicine, New Orleans, Louisiana

Those viruses which produce diseases in which eruptive lesions of the skin and, or, mucous membranes constitute either the only or predominant manifestation are generally considered as dermatropic. Implying a more or less selective affinity for the cellular elements of the skin and mucous membranes the term cannot be accepted as strictly definitive. A few of the members of this group are perhaps sharply limited in their individual cellular tropisms and as such should be designated more specifically as epitheliotropic. The majority, however, exhibit a wider range of cellular affinities than is implied in the term dermatropic.

The nomenclature applied to this group has given some occasion for discussion. The terms Chlamydozoa and Strongyloplasm introduced by von Prowazek (1) and Lipschutz (2) are no longer in current use. More recent knowledge regarding viruses would seem to confer a greater pertinence to the concepts upon which these terms were based than for a time was attributed to them. The original observation establishing the presence of elementary bodies in vaccinia was made by Buist in 1886 according to Gordon (3) and Mackie & Van Rooyen (4). Since Paschen's observations (5) reported in 1906 were more widely recognized, the designation *Buistia pascheni* was proposed for vaccinia virus (4).

Goodpasture's proposal (6) to apply the generic term Borreliota to the pox group of viral agents recognized Borrel's original (7) description in 1904 of the elementary corpuscles of fowl-pox. This terminology has found current acceptance in the 6th edition of *Bergey's Manual of Determinative Bacteriology*. Under the Order Virales the Family Borreliotaceae designates the "Viruses of the Pox Group, inducing diseases characterized in general by discrete primary and secondary lesions of the nature of macules, papules, vesicles or pustules" (8). This division includes five genera to which 21 human, mammalian and avian viral species are assigned.

Based upon the general characteristics of the clinical lesions produced by the viral species included in the group, this classi-

fication has the advantage of not being committed to their individual cellular tropisms. The nomenclature devised to designate each genus with its included species has much to recommend more general adoption. Further definitive characterization of the biochemical, biophysical, as well as the antigenic constitution, of these viruses is not only essential for the purpose of classification but is intimately concerned with a better understanding of cytotropism. Exclusive intracellular multiplication is not limited to viruses. However, some of the agents to be treated in this discussion are perhaps most ideally suited to an intensive analysis of this phenomenon. Its further elucidation is of paramount importance for the development of a better understanding of the pathogenesis of a great variety of infectious diseases.

It will not be possible to review adequately the experimental investigations which in recent years have been performed with the viruses included in the family Borreliotaceae. Some of the most extensive work in the virus field has been devoted to a few members of this group. Others have received scant attention and some have been neglected altogether. The selection of the topics for discussion must necessarily be restricted. The bibliography will be limited to publications dealing with work indicating the general trend of research activity. An attempt will be made to emphasize what appears to be germane to progress in knowledge as it applies to the virus field in particular and to the problems of infectious diseases in general.

The Pox Viruses

The pox viruses as a group share many features in common, and little difficulty is encountered in considering them within a well-defined category. Within rather narrow limits of size and morphology there is a marked uniformity among the elementary bodies which represent the active agents of these diseases. In each case ectodermal epithelium is parasitized either exclusively or during the eruptive stage of the infectious process. The proliferative response of the susceptible epithelium producing the typical pock-like lesions is basically the same. In the focal lesions of each of the variety of diseases of this group the parasitized cells are characterized by the presence of intracytoplasmic inclusions of which the elementary bodies are essential components.

Elementary bodies.—Elementary bodies have been demon-

strated in constant association with the lesions of the skin and in a great variety of tissues and organs of the different species found susceptible to infection with the pox viruses. That they represent the infectious unit has been well established. Woodruff & Goodpasture's classical experiments with the virus of fowl pox (9) provide much of the fundamental evidence for this conclusion. Eagles & Ledingham (10) and Craigie (11) confirmed the original observations of MacCallum & Oppenheimer (12) concerning the infectivity and particulate nature of the infectious agent of vaccinia. Quantitative studies have (13) provided direct correlation between the numbers of elementary bodies with the infectivity of a given suspension. The application of statistical methods (14) has demonstrated that under ideal conditions a single elementary body could induce infection. Under usual conditions of infection the situation is much more complex in that a minimal infective dose usually consists of numerous elementary bodies (15, 16). Many other important considerations enter into the relation of the numbers of elementary bodies capable of inducing infection. Host susceptibility (17), variations in inherent virulence of vaccinia elementary bodies (18), and the source of the virus and species used for test inoculation (19) are of importance. Smaller inocula of vaccinia elementary bodies have been found to be relatively more effective than larger ones (20, 21).

Morphology and size.—By means of the older staining methods introduced by Paschen, Morosow, Herzberg, and others, the elementary bodies in smear preparations appear as small round granules occurring singly, in pairs, short chains, or clusters. Direct examination of fresh preparations by ordinary microscopy, by darkfield illumination, and by ultraviolet photography all seem to indicate that the elementary bodies are spherical in shape.

The first electron micrographs of the elementary bodies of vaccinia were described by Green, Anderson & Smadel (22). Many important details of structure have been brought into sharp relief by the shadow casting techniques introduced by Wyckoff & Williams (23). These observations performed with vaccinia, variola, and fowl pox elementary bodies substantiate earlier evidences indicating their marked similarity to one another. In electron micrographs they appear to present six rectangular surfaces so arranged that their three-dimensional shape is brick-like. Internal structures in the form of a central mound-like elevation of increased

density surrounded by other smaller areas of similar density are observed. A limiting outer membrane is also apparent. The particles are surrounded by a sticky substance and appear in many instances to be joined at their corners. Evidence of unequal division of the elementary bodies is also obtained. These observations strongly support the conclusion that the pox viruses possess morphological characteristics that are similar to those of the bacteria (22, 24, 25).

The evidence thus far available, however, does not permit of final conclusions regarding the actual morphological structure of the elementary bodies. The discrepancy between the earlier observations indicating a spherical configuration and the more recent evidence of a brick-like shape are as yet not resolved. Electron micrographs have been interpreted as indicating a more or less cylindrical shape (26).

The size of the elementary bodies of the pox group has been estimated by various procedures. The earlier impressions of Buist obtained by direct microscopy gave an estimate of about 150 mμ. Ultrafiltration and ultracentrifugation methods have variously provided results indicating a range from 125 to 250 mμ. The most exact estimation derived from calculations which take the sedimentation constant into consideration give a figure of 236 mμ. for the elementary bodies of vaccinia (27). Direct measurements of electron micrographs of shadowed elementary bodies of variola and vaccinia involving a consideration of the brick-like shape under these conditions indicate an average size of 244 by 302 mμ. (24).

Elementary body suspensions.—Much of the knowledge regarding elementary bodies has become available through the development of methods for preparing suspensions of vaccinia virus almost completely free from extraneous material. It must be taken into account that those methods which provide the greatest concentration of elementary bodies are achieved with strains of vaccinia virus which have been adapted by repeated rapid passage in specific cells of a particular host. Craigie's elementary body suspensions are derived from a strain of vaccinia virus which has been adapted to extremely rapid multiplication in the superficial epithelium of the rabbit skin (11). Smadel & Wall (28) obtained their suspensions from infected chick embryo chorio-allantois. Such strains are markedly homogeneous as judged by their restricted

cellular tropisms. Analyses of elementary body suspensions prepared from calf lymph (29) or from sufficiently large quantities of human variola crusts if compared with results obtained with the more homogeneous strains would perhaps provide data applying to problems regarding differences in virulence, pathogenicity, and antigenicity.

Studies of this nature require much more vigorous pursuit. They should be applied to the various strains of variola which may be derived from hemorrhagic, severe, and mild smallpox and from alastrim. The various distinct strains of vaccinia provide opportunities for comparison. This also applies to fowl pox, pigeon pox, canary pox, and ectromelia. Each of these virus strains is readily adapted to propagation in the chick embryo by either chorio-allantoic or intra-amniotic propagation and thus a uniform substrate for comparative purposes is provided.

Chemical composition; physical properties; metabolism of elementary bodies.—The reports of Hughes, Parker, & Rivers (30), Hoagland *et al.* (31 to 34), Smadel *et al.* (35), MacFarlane *et al.* (36, 37), and McFarlane *et al.* (38) should be consulted for an account of the chemical composition and physical nature of the elementary bodies of vaccinia. These studies give support to the judgment that vaccinia virus possesses many of the properties of chemical composition attributable to bacteria. The density of vaccinia elementary bodies lies within the range of 1.12 to 1.18. A single elementary body has been calculated to weigh 5.2 to 5.34×10^{-15} gm. (15, 39). Elementary bodies of vaccinia exhibit extremely slight metabolic activity which is considered as compatible with survival rather than multiplication (40, 41). Phosphatase, catalase, and lipase activity have been demonstrated (36). Hydrolysis of nucleic acid can be shown but dehydrogenases, zymohexase, A and B glucosidases, nucleosidase, and enolase seem to be lacking (37).

Inclusion bodies.—Considerations regarding the nature of the inclusion bodies and their relationship to elementary bodies of the pox viruses have been involved in the problems of the etiology of viral diseases (42). The experiments of Woodruff & Goodpasture resolved many of the primary difficulties. The infectivity of the individual inclusions of fowl pox was demonstrated by micromanipulation experiments. The elementary bodies were shown to comprise essential constituents of the inclusion. Other structural components, such as a surrounding encapsulating material which

is partly lipoidal in nature, and a lipoprotein matrix in which the elementary bodies are embedded, were clearly identified (19.)

Observations on the structure of the inclusion bodies of vaccinia in the infected chorio-allantois indicated that although no encapsulating substance was present, the elementary bodies were the chief constituent (43). Micromanipulation experiments with molluscum contagiosum inclusions essentially confirmed the previous observations on fowl pox (44). The use of the "Ultrapak" system of microscopic illumination for the direct examination of infected cells *in situ* added supporting evidence (45). The more recent electron micrographs of variola, vaccinia, and fowl pox strikingly illustrate many of the features brought out by the earlier studies (24, 25).

The presence of the inclusion bodies of vaccinia within infected tissue culture cells has been repeatedly demonstrated (46, 47, 48). The successive stages of their development within rabbit corneal epithelium grown *in vitro* have been described in detail. The inclusion bodies were found to consist of elementary bodies within a matrix. They apparently represent an obligatory stage in the multiplication of the elementary bodies (49). A variety of complex morphological changes which inclusions exhibit following the entry of elementary bodies into the cytoplasm have been described by observations made on epithelial cells detached from the infected rabbit cornea (50). These studies also presented evidence that elementary bodies were found to multiply and form colonies within leukocytes (51).

The identification of the intranuclear inclusions most typically found in the lesions associated with variola, and occurring to some extent in alastrim and in what has been described as para-vaccinia, is uncertain. They have been considered as nonspecific changes incident to the infectious process. It is not known whether they represent parasitization of the nucleus by variola or indicate the presence of another of the viruses which are known to produce intranuclear inclusions.

These varied studies devoted to the nature of elementary bodies and the development and structure of the inclusion bodies of the pox group have not provided satisfactory evidence concerning the first stages of pathogenesis which involve the actual mechanism of infection. Careful consideration has been given to these problems of pathogenesis in discussions by Goodpasture (42, 52),

Rivers (53), and Doerr (54). The factors which determine the entry of virus particles into susceptible cells are not clearly definable. These considerations entail many of the basic manifestations of specific cytotropism, i.e., the parasitic adaptation of particular viruses to the internal environment of certain cell types. The problems are still more complex in regard to cell to cell transmission. The factors operative in the fixation of virus from the blood or lymph stream by susceptible cells during the stage of generalization are as yet not well understood.

Antigenic structure and immunity reactions.—More detailed accounts of the immunity responses to the pox viruses, in particular vaccinia, and the variety of serological reactions applicable to their analysis are available in River's textbook (55) and the reference work of Van Rooyen & Rhodes (56). With the use of elementary body suspensions of vaccinia the well-established serological reactions used in bacteriology have been intensively investigated. In general these studies demonstrate the applicability of the classical *in vitro* tests to an analysis of the antibody response to the pox viruses. Burnet's introduction of the pock counting technique on the chorio-allantois has placed the quantitative analysis of the virus neutralizing capacity of immune serum on a more accurate basis than was formerly available (57). Because of the more uniform susceptibility of the embryo to the various members of the pox group this method allows for further investigations of cross-immunity reactions. The method was found to be particularly useful for determining the neutralizing capacity for variola virus of the sera of individuals immunized by Jennerian prophylaxis (58).

A new approach to the analysis of the relative roles of cellular and humoral factors operating in immunity to virus infections was introduced by Goodpasture & Anderson (59). Skin grafts from fowls immune to fowl pox were transferred to immune and nonimmune adult hosts and also to the chorio-allantois of the susceptible chick embryos. In the nonimmune host the grafts from immune fowls were completely susceptible to reinfection. Transferred to immune hosts their insusceptibility was retained. *In vivo* tests, involving the passive transfer of whole blood, serum, plasma, and plasma-leukocyte mixtures, demonstrated that a limited but distinct inhibitory action on reinfection could be produced. Whole blood and plasma-leukocyte mixtures exerted a greater modifying

activity on the extent and character of the lesions than did serum alone. It was concluded that humoral antibodies seemed to represent the most potent agents in the acquired immunity of the cutaneous epithelium to fowl pox infection. The experiments indicated that other factors as yet not clearly discernible are involved (59). In some way or other the host as a whole seems to contribute to resistance to reinfection.

Hemagglutination and hemagglutination inhibition.—The agglutination of the erythrocytes of chickens and of several other species first described for influenza virus [Hirst (60) McClelland & Hare (61)] has also been demonstrated for vaccinia and variola (62, 63). This reaction produced by the viruses of the pox group appears to be of a different order than that which occurs with influenza and Newcastle viruses. The red cells of only about 50 per cent of fowls are susceptible. The reaction takes place best at 37°C. as contrasted with the lower temperatures effective with influenza. Elution of vaccinia virus from agglutinated cells does not occur (64). Red cells from which the receptors for influenza virus are removed are susceptible to agglutination by vaccinia virus (65).

Soluble substances, rather than the virus particles themselves, apparently are responsible for hemagglutination. Their action is similar to that of lipid complexes, possibly phospholipids which can be derived from the normal chorio-allantois and other animal tissues. Hemagglutination by vaccinia is perhaps mediated by these substances which enter into a rather stable combination with one or more antigenic complexes derived from the viruses. *Clostridium perfringens* toxin and cobra venom are capable of inactivating the lipid complexes which appear to be essential to the reaction (66).

Vaccinia antiserum specifically inhibits hemagglutination. The reaction has been adapted to the study of the serology of the immune response to smallpox vaccination (62). It has proved to be of considerable interest in providing evidence that there is a definite biological relationship between vaccinia and ectromelia. Cross-immunity between these agents can be demonstrated, and it has been proposed that ectromelia may be considered as the virus of mouse pox, belonging to the pox group of viruses (65).

The viruses of the pox group are generally considered as capable of stimulating a long lasting if not a permanent immunity. This impression has been largely based on the widespread practical

application of vacccination as a protection against smallpox. When considered critically it is quite apparent that much information is still required for an adequate understanding of the factors which are operative in this phenomenon. No positive statements can be made as to the approximate range of the duration of the protection afforded by a primary vaccinal reaction against variola. Much still remains to be learned as to the persistence of circulating antibodies in relation to the duration of effective resistence. The nature of the accelerated or vaccinoid reactions, especially in regard to the relative roles of tissue hypersensitivity and circulating antibodies, requires further elucidation. Although these considerations may appear to be primarily of academic interest, recent experience with the occurrence of smallpox in military personnel exposed to epidemics and the sudden demand for mass immunization of a large civilian population during the New York City episode emphasizes their practical import (67, 68, 69). There is as yet no satisfactory resolution of the problems regarding the basic mechanism of resistance to viral infections as they pertain to the importance of humoral antibodies as compared with general and specific reactions of cells and tissues of the immune host. Further applications of the newer techniques introduced during the past 10 years should provide improved opportunities toward a solution of some of these problems.

Strain variants of the pox viruses.—The existence of several distinct forms of clinical variola in man is presumably due to the spontaneous development of strain variants. The discovery of inherent characteristics which would account for the wide range of virulence and pathogenicity exhibited by hemorrhagic smallpox, variola major and minor, and alastrim should prove to be of great value. Experimental variola is most readily produced in monkeys. Recent studies and the numerous earlier investigations of this type have been largely concerned with comparisons of variola with vaccinia (70, 71). Comparisons of morphological and tinctorial features of the inclusion bodies of variola major and alastrim in sections from lesions of the human and experimental diseases may have diagnostic significance but present no evidence for basic differences (72).

The introduction of the chick embryo chorio-allantois for the propagation of variola virus presents opportunities for comparative studies of the basic behavior patterns of strains of variola

virus derived from different forms of the disease (73, 74). The method has been found admirably suited to the diagnosis of variola. It is apparently much more sensitive than the Paul test, especially for the diagnosis of mild smallpox (75, 76).

In the chorio-allantois the strains of variola studied remain remarkably stable upon continued passage. Infections with virus derived from smallpox cases in the United States and with one of Chinese origin were restricted to involvement of the ectodermal epithelium of the chorio-allantois. Generalization to the embryo did not take place (75, 77). Smadel, however, isolated a strain which killed embryos with great regularity (78). In the chorio-allantois variola virus has shown no tendency towards transformation to vaccinia (77).

The question of the derivation of vaccinia virus from variola in man has in no way been satisfactorily settled. The similarity of antigenic structure of the various strains of variola and vaccinia is readily demonstrated by cross-immunity and by the complement fixation, agglutination, and neutralization tests. Minor differences are observable and the immunity is not completely reciprocal (79).

There is no substantiation of the hypothesis (80, 81) that vaccinia consists of equal parts of variolar and vaccinial components or that freshly isolated variola contains only a small proportion of vaccinial elements which are selectively increased on animal passage. It is difficult to disregard the evidence indicating that by successive passage in monkeys, rabbits, or calves, or by a combination of passages in one or more of these hosts, variola has been transformed into vaccinia. Whether the terminal vaccinia thus derived originated from the initial variola still remains to be demonstrated. These investigations have usually been performed in smallpox vaccine laboratories and the possibility of accidental introduction of vaccinia cannot be considered to have been excluded. Intensive efforts to induce this transformation by the traditional methods by the author of this review supports the hesitancy of others (77, 82) in accepting as proven what is generally considered as a well-established fact.

Each of the many variants of vaccinia is essentially a laboratory strain. They have been produced chiefly in rabbits under varying conditions of passage in the skin, or by intratesticular, intracerebral, or a combination of all three means of passage. The several strains of vaccinia which are propagated in calves

for the production of vaccine used in Jennerian prophylaxis are considered as dermal. There is considerable variety in the empirical methods followed in the so-called "fortification"of these strains by the addition of virus derived from human and rabbit vaccinia to the seed used to infect calves. Whether or not these variations in procedures effect differences in antigenic quality of vaccines produced in different laboratories has not been determined. They have been found to differ from one another in their capacity to parasitize cells of mesodermal origin as determined by their behavior in the chorio-allantois. Some are strictly epitheliotropic. Others show a marked range in their affinity for mesodermal fibroblasts and capillary endothelium (82). It is suspected that those strains which are more frequently "fortified" are more heterogeneous in their cellular affinities as contrasted with strains with a homogeneous affinity for epithelial cells only. These differences perhaps account for the marked variations in the severity of the vaccinal lesion in man produced by strains from different laboratories.

Calf lymph strains of vaccinia with an initial affinity for mesodermal cells upon repeated passage in the chorio-allantois of chick embryos become exclusively epitheliotropic (83). They produce a much milder and more superficial infection in rabbit skin than the calf vaccines from which they are derived. In this respect they resemble the strains produced by rapid passage in rabbit epithelium which have been so sucessfully used for the production of elementary body suspensions. Strains propagated in tissue culture also possess this characteristic (54). In human infection with the tissue culture virus and with chick embryo propagated virus the vaccinal lesion is much milder than that usually produced by calf propagated virus (83, 84, 85). The milder character of the disease produced by these strains may in part be attributable to the fact that they are free from bacterial contaminants. It is, however, much more likely that their homogeneity, as evidenced by affinities restricted to epithelial cells only, is responsible for the relative mildness of the lesions.

The immunity induced by tissue culture vaccinia appears to be less stable as tested by revaccination with potent calf lymph than that produced by calf lymph itself (86). As measured by the induction of vaccinia and variola neutralizing antibodies, vaccination with chick embryo virus is comparable in its effectiveness to virus grown in calves. Neither does there seem to be any

difference in immunity as measured by revaccination with potent calf lymph (83). These observations, however, were made only at a one year period following the primary vaccination. No conclusions can as yet be made as to the actual length of time during which effective immunity to smallpox may be expected to result from vaccination with these strains.

These considerations must be taken into account not only in an evaluation of the effectiveness of smallpox vaccine prepared from chick embryo virus but also in relation to the entire problem of Jennerian prophylaxis regardless of the method of virus propagation. There is no question regarding the capacity of any type of virus which is capable of producing typical vaccinia in man to stimulate the development of antibody response and to induce immunity to revaccination for periods of perhaps several years. Less definite information is available regarding the actual duration of effective resistance to smallpox.

During the early stages of human vaccinia infection the virus generalizes and has been demonstrated in the blood and in the nasopharynx (87, 88). It is not unlikely that the stimulus to a more effective and durable resistance is greater under these circumstances than when this event does not occur. Whether or not the more homogeneous strains which apparently infect only epithelium of the skin, maintained either by calf, rabbit, sheep, chick embryo, or tissue culture methods, are capable of inducing generalization, or whether this property is confined to heterogeneous strains which have the capacity to infect cells of mesodermal as well as epithelium of ectodermal origin, remains to be demonstrated. If generalization of vaccinia is not the mechanism whereby adequate immunity to variola is produced, it still remains to be proven whether a superficial epithelial infection can accomplish this, or whether perhaps other factors are concerned.

The so-called neuro-testicular strains are characterized by a high degree of affinity for cells of mesodermal origin and especially for endothelial cells (89, 90). This is perhaps also true of spontaneous rabbit pox and the heat resistant strain of Armstrong (91, 92). Fowl pox virus which under usual circumstances of infection is strictly epitheliotropic may, by intracerebral passage in chicks, produce a stable variant which has affinities for mesodermal and especially endothelial cells comparable to that observed in strains

of neuro-vaccinia (93, 94). In reference to the problems outlined above such variants deserve further study in relation to their immunity-stimulating capacity.

HERPES SIMPLEX VIRUS

As one of the more common agents of human infection the virus of herpes simplex has attained for itself a highly advantageous degree of parasitism. Without being unduly injurious to its host, mechanisms have become established which serve to maintain it successfully (95). Herpes labialis, the common cold sore or fever blister, is the most frequently encountered clinical form of this infection. This recurrent or secondary form of the disease takes place in individuals who have apparently developed an altered reaction to the infectious agent induced by the primary disease usually acquired early in life. The primary disease manifests itself as an acute gingivo-stomatitis occurring in individuals who have developed no active specific resistance (96, 97). Other forms of infection, eczema herpeticum, herpetic conjunctivitis and kerato-conjunctivitis, herpes gentitalis, and rarely herpetic meningoencephalitis are also recognized.

The virus particles of herpes have a diameter of 100 to 150 mμ. as determined by gradocol membrane filtration (98). Ultracentrifuge studies indicate a particle size in the range of 190 to 220 mμ. (99). Elementary bodies, in stained smear preparations visible by ordinary microscopy, have been described (100) but their identity has not been satisfactorily established. Successful electron micrographs of herpes virus have not as yet been published.

Great difficulties are encountered in obtaining relatively pure suspensions of herpes virus comparable to those prepared with vaccinia. It has therefore not been possible to obtain satisfactory data regarding its physical and chemical properties. Such preparations if available would be of great value in the development of knowledge regarding this virus. Chorio-allantoic lesions (101), allantoic or amniotic fluids from infected embryos should prove adaptable for the preparation of herpes virus suspensions.

Tissue suspensions containing the virus will fix complement in the presence of specific antibody (102). Numerous workers have demonstrated the virus neutralizing activity of immune serum. The pock-counting method of Burnet is particularly well suited

for quantitative estimations of the serum neutralizing capacity (103, 104). Hemagglutinin reactions have thus far not been demonstrated with herpes virus.

Strain variants of herpes simplex are well recognized. The highly neurotropic H. F. strain maintained by constant intracerebral passage in rabbits or mice when inoculated on the rabbit cornea almost invariably invades the central nervous system by way of the sensory division of the fifth cranial nerve. When peripheral infection is established elsewhere with this or certain strains there is a marked tendency to spread to the central nervous system along the fibres of the regional sensory, motor, or sympathetic nerve supply (105). There is a marked variation in the neurotropic qualities of strains of virus derived from human infections. Some are capable only of producing keratitis and of these only certain ones will be found infectious for rabbit or guinea pig skin. Strictly epitheliotropic strains are difficult to maintain by passage in the skin of rabbits or guinea pigs.

Prolonged propagation by passage in the chorio-allantois induces distinct variants. After several passages the H. F. strain produces only a keratitis without clinically recognizable encephalitis (106). Strains derived from cases of gingivo-stomatitis have been found to lose infectivity for rabbit cornea after several passages in the chorio-allantois (82).

Antigenic differences have been observed by means of serum neutralization tests between strains derived from cases of vulvovaginitis, other strains of human origin, and the H. F. strain (107, 108). The application of other serological techniques, such as complement fixation or the pock-counting method, will undoubtedly reveal other antigenic distinctions.

Active infection with herpes simplex is almost invariably associated with the presence of intranuclear inclusion bodies within the parasitized cells (109). Their development has been observed in tissue culture (46), in the infected chorio-allantois (110), in grafts of human chorion and amnion on the chorio-allantois (111), and in the cells of various tissues and organs of the developing chick embryo. There is good evidence to indicate that the intranuclear inclusions represent localized concentrations of the virus. By micromanipulation single washed inclusions have been found capable of inducing herpetic keratitis (112). Microincineration and ultracentrifuge studies give indications that these structures do

not represent degeneration products derived from chromatin (113, 114).

Histological studies support the judgment that the intranuclear inclusions represent the intracellular site of virus multiplication. A progressive development in size from extremely small granules to finely granular structures are described in the infected chorio-allantois (110). Nicolau's observations on their structure indicates that they are composed of elementary bodies from 100 to 500 mμ. in diameter (115). Whether or not the nucleus is the only site of virus multiplication cannot be definitely stated. The fact that vaccinia and herpes together can parasitize individual cells, each producing typical inclusions, is at least suggestive that these specific intracellular localizations are characteristic of each virus (116, 117).

The pathogenesis of experimental herpetic infection has received considerable attention by numerous investigators. Of fundamental importance to the development of the concept of cytotropism in reference to the involvement of the central nervous system with viruses which utilize nervous pathways in their centrifugal spread, have been those studies concerned with herpetic infection of the rabbit (105). More recent work on this infection in chick embryos and in mice has provided further evidence of the value of this virus as a model for a study of the pathogenesis of those viral diseases in which the central nervous system is involved by way of nerve tracts (106, 118 to 121).

It is now quite generally accepted that in human beings herpetic infection is usually acquired early in life as a primary self limiting disease. Most instances of so-called aphthous stomatitis which occur most commonly between the ages of one and five years have been demonstrated to represent the first infection with herpes virus. A passive humoral immunity, presumably acquired *in utero*, apparently is effective in protecting infants during the first six to twelve months of life (96, 97). Herpetic gingivo-stomatitis usually occurs when the passively acquired antibodies have disappeared from the circulation. Specific virus neutralizing antibodies develop during the course of the infection. The occurrence of the primary disease probably accounts for the fact that a significant proportion of the adult population possesses a high level of antibodies (122).

These observations regarding the occurrence of primary her-

petic infection and the development of circulating antibodies early in the life of most individuals are basic to a consideration of the problem of herpetic infection. Although it is obvious that man constitutes the only reservoir it is not too clearly demonstrated as to how the virus is transmitted. In the majority of cases of primary herpetic stomatitis evidence is available for more or less direct or indirect contact with individuals with gingivo-stomatitis or recurrent labial herpes.

The factors operative in recurrent herpes are still to be defined. Circumstantially numerous conditions such as fever, intercurrent bacterial infections, physical irritation, etc. are implicated as provocative stimuli. This raises the problem as to the endogenous or exogenous source of the infectious agent. Evidence has been presented to indicate that herpes virus is maintained in a latent state. Its distinct neurotropic affinities have given occasion for postulating that the local sensory ganglia supplying the affected area represent the site where the virus is maintained (105). Others suspect that the epithelial cells at sites of recurrence harbor the virus (95). Substantiation of either of these, or any other hypothesis, would demand much exacting work to demonstrate presence or absence of the virus in various sites over long periods of time in individuals who are, as well as those who are not, subject to recurrent herpes. In this respect it has not as yet been proven that the methods available for the isolation of the virus are sufficiently sensitive to detect small amounts of virus in the latent state. A limited amount of experience indicates that the chorio-allantois is more sensitive for this purpose than is the rabbit cornea (82).

Insufficient data are at hand to allow for definite conclusions regarding the role of antibodies in herpetic immunity in relation to recurrent infection. It is not known whether the primary infection provides the necessary stimulus for maintaining a persistent level of antibody, whether the persistence of the virus in the latent state maintains an infection immunity, or whether repeated exposure with minimal or subclinical infection maintains the antibody level. These factors must be considered in respect to the susceptibility of local cells in the presence of a high antibody level. This circumstance apparently obtains in recurrent labial herpes and in occasional cases of recurrent-herpetic stomatitis. Certain observations suggest that a blood-epidermis barrier is involved in this peculiar local tissue susceptibility in the pres-

ence of humoral immunity (121). The influence of a strong hypersensitivity component in the pathogenesis of recurrent herpes is suggested by its accelerated character as compared with the primary infection. Immune individuals have been shown to react to intradermal injection with inactivated herpes virus (123). This test may prove of help in an evaluation of the hypersensitivity involved.

Although not a severe disease, recurrent herpes is sufficiently annoying to justify further attempts to obtain an effective safe immunizing agent. Chick embryo adapted strains may prove to be useful for this purpose.

Measles

Although, like herpes simplex, measles is one of the most common of human infectious diseases, it has presented a much more difficult problem to the investigator. The viral etiology of this disease was established almost 30 years ago with the demonstration of the susceptibility of the monkey to infection with Berkefeld filtrates of nasopharyngeal secretions from human cases (124). The monkey has not proved too suitable for definitive studies since it is relatively insusceptible, and usually only a few of the characteristics of the disease can be regularly reproduced. Others of the usual laboratory animals have proven unsatisfactory. Successful propagation in limited serial passage in tissue culture has been reported (125, 126). In recent years a certain amount of success has been obtained with propagation of this agent in the chick embryo (127, 128).

Very little information has been established regarding the properties of measles virus. It is known to be filterable through Berkefeld N and Seitz E. K. filters, but its size and morphology have not been determined. Elementary bodies have not been conclusively demonstrated. The observations of Broadhurst on the presence of inclusions and elementary bodies in mononuclears of the blood and epithelial cells from Koplik's spots and from the nasal mucosa have not been confirmed (129).

The cytology of the rash in measles indicates that in the exanthematous stage the epithelium of the skin is vulnerable to the presence of the virus. Vacuolation and necrosis of epithelium with subsequent vesicle formation and desquamation are the typical responses (130). The presence of intranuclear, as well as intracy-

toplasmic, inclusion bodies has been variously reported. They have not been consistently observed or characterized and their identity is highly problematical. Until this disease can be successfully reproduced experimentally or the behavior of the virus in the natural disease in man can be more definitely ascertained by means of adequate isolation and identification tests, many of the stages in the pathogenesis of measles must remain undefined.

The apparently encouraging beginning obtained with the propagation of measles in the chick embryo has not been productive of much data essential for a better understanding of this agent. The method deserves more vigorous pursuit. It is quite possible that more readily adaptable strains may be encountered.

Virus from early passages in the chick embryo is reported to be capable of producing a slightly modified disease in monkeys and man, while that from later passages produces a markedly modified disease, or none at all. The production of a modified disease induces an increase in resistance to the natural disease. It is interesting to note that the more mild type of disease induced by virus from later passages stimulates a partial and perhaps temporary resistance whereas the immunity from the naturally acquired disease seems to be a permanent one (128).

Speculation in this regard suggests that naturally occurring measles virus is perhaps heterogeneous in its cellular tropisms with a marked capacity for generalization. Propagation in embryonic tissue may, as noted in vaccinia and herpes, favor the development of more homogeneous strains with enhanced affinities for only one cell type, presumably epithelium. In the natural disease involvement of the skin appears to represent one of the later phases of the infectious process. During the pre-exanthematous period cells of other tissues are quite likely parasitized. This circumstance constitutes a much more profound experience for the host and should serve as a much more adequate stimulus to the immune response than does the involvement of superficial epithelium alone. Whether the generalization of the virus accounts for the persistence of resistance, or whether in some way measles is retained in a latent form establishing an infection immunity remains to be determined.

German Measles (Rubella)

The demonstration that the occurrence of rubella in pregnant women has a profound effect on the fetus *in utero* (131), and under

these circumstances produces a variety of congenital malformations, has placed this otherwise mild exanthematous disease of childhood in a prominent position. The viral etiology of the disease was established by its transmission to children with filtered nasal washings (132). Habel (133) has recently confirmed earlier observations of Hess (134) on the susceptibility of rhesus monkeys.

Except that it is filterable through Berkefeld W and N or Seitz E. K. filters nothing is known regarding the properties of the virus. According to Habel (133) it has been propagated in the chorioallantois of chick embryos. Although no visible lesions were produced rubella was successfully transmitted to the monkey after five membranal passages. These observations, if confirmed, contain enticing possibilities, not only for propagation of the virus, but especially for studying the effect of infection in this embryonic host. Should the virus prove capable of proliferation in chick embryos during the first trimester of development, opportunities would be available for a study of the pathogenesis of the congenital malformations which occur when rubella is contracted during pregnancy.

This behavior of rubella obviously emphasizes the need for further investigation of the effect on the fetus of other viral diseases acquired during pregnancy. Cellular specificity does not develop to a pronounced degree in the early stages of embryonic life. During these stages cells and tissues of many types seem to possess a broad biological substrate to which many viruses are adapted (135).

Varicella-Herpes Zoster

Numerous observers have presented evidence which lend support to the suggestion proposed by von Bokay in 1888 (136) regarding the possible relation of varicella to herpes zoster. A striking similarity exists between the gross features and cytopathology of the cutaneous lesions of both diseases. An impressive amount of epidemiological evidence has been presented indicating that the etiological agents are probably identical (137). However, the critical demonstration of their relationship to one another still remains to be made. Until the agents of these diseases can be successfully propagated under the experimental conditions the evidence regarding the relation of varicella to herpes zoster to herpes simplex is only circumstantial.

The reproduction of varicella in human beings by means of bacteria-free vesicle fluid and the production of local vesicular lesions in young children with vesicle fluid from herpes zoster provided evidence for the viral etiology of these diseases (138, 139). Herpes zoster has been claimed to be filterable through Berkefeld filters (140). The presence of intranuclear inclusions in the epithelial cells of the cutaneous lesions in both varicella and zoster and in the generalized lesions of occasional cases of fatal varicella is further indicative of the viral nature of these diseases (141).

Elementary bodies have been described for each and are of similar size, estimated at from 145 to 250 mμ. in diameter. Recent electron micrographs of vesicle fluid obtained during the first 24 hr. after the appearance of the vesicles demonstrate the striking morphological similarity of the elementary bodies of varicella and zoster (24, 142). The bodies appear brick shaped, singly, in pairs, and clumps often surrounded by a sticky matrix. The average size by electron micrograph measurement of varicella is 208 by 240 mμ., that of herpes zoster 186 by 288 mμ.

The intranuclear inclusion bodies of varicella are morphologically similar in all respects to those of herpes zoster. These structures in all probability represent intranuclear aggregations of elementary bodies. This supposition still requires adequate demonstration. The clumps of elementary bodies embedded in a sticky matrix as seen in electron micrographs (24, 142) resemble those of vaccinia and fowl pox (24, 25) which definitely suggests that the inclusions largely consist of elementary bodies similarly held together.

No conclusive evidence has been presented that animals other than man are susceptible to infection with varicella or herpes zoster. Attempts to propagate these viruses in the chick embryo have thus far been unsuccessful. Inoculation of the chorio-allantois has for this reason been proposed as a differential diagnostic test between variola and varicella (58). Typical intranuclear inclusions have been obtained following inoculation of human skin grafts on the chorio-allantois. Human epithelium only was thus affected; the membranal ectoderm surrounding the graft appeared to be insusceptible (143). Nevertheless, it seems quite probable that these viruses can be adapted to propagation in the chick embryo. The observations of Rake *et al.* (142) emphasize the importance of ob-

taining fluid in the very early vesicle stage. At that time elementary bodies are abundantly present but seem to disappear quite rapidly within relatively few hours thereafter.

Serological reactions with varicella and zoster have been confined to agglutination of the elementary bodies with human convalescent or immune serum. Reciprocal cross agglutination apparently occurs but these reactions cannot be considered as conclusively establishing their antigenic relationships (144). Further investigations await the successful propagation of these viruses in a susceptible experimental host.

In many respects the pathogenesis, immunology, and epidemiology of these infectious diseases are the same as those encountered in herpes. Not only is the relationship of varicella to herpes zoster of more than theoretical interest, but many of the features characteristic of these viruses and the lesions they produce are similar to those encountered with herpes simplex virus. The formulation of the problems involved as presented 20 years ago by Goodpasture (105) to a large extent still awaits solution.

In this discussion it has been possible to consider relatively few of the viruses which have been for one reason or another designated as dermatropic. Most of the attention has been directed toward those which are more commonly encountered as agents of human disease. It must be emphasized however that besides fowl-pox and ectromelia there are a number of viruses natural to animals which produce eruptive diseases and which lend themselves to and deserve much more widespread interest from investigators of the problems of infectious disease, especially as they relate to the host cell-parasite relationship.

Much progress has been made since the demonstration of the existence of these minute agents of disease by Iwanowski and Loeffler & Frosch some 60 years ago. This is particularly true of the development during the past 15 years of methods whereby several viruses have been visualized and characterized morphologically and many of their essential properties defined. The basic nature of the intimate parasitization of susceptible cells and the behavior of viruses in respect to various cell types of the host as a whole during the various stages of the infectious process, and the effect of these experiences on subsequent resistance and reinfection have proven to be much more elusive.

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FOOD MICROBIOLOGY

By H. O. Halvorson

Department of Bacteriology and Immunology, University of Minnesota Minneapolis, Minnesota

The problems facing the food bacteriologist are much the same from year to year; he must detect and take steps to prevent contamination of food with undesirable organisms; sterilize canned goods effectively without impairing quality; improve sanitary conditions in food manufacturing plants; control the growth of microorganisms by refrigeration, freezing, desiccation, or by use of inhibitors. In reviewing literature on food bacteriology for any period, articles will be found dealing with each of these problems. The type of food which receives the bulk of attention will vary from time to time, but the overall problems remain the same. In food bacteriology, as in other fields of bacteriology, there has developed a degree of specialization, and in a short review of this kind, it is impossible to cover adequately all the specialities in the field. Dairy bacteriology, although a branch of food bacteriology, is in reality a specialty of its own and should be reviewed as a specific topic. It has, therefore, not been covered in this review. Also for the sake of brevity, the period has to be limited. The author, therefore, has limited the review to publications which have appeared during the past two years.

Egg Products

Liquid egg products have become important food items during the past decade and their use has increased considerably. These products are easily contaminated and provide an excellent medium for the growth of microorganisms. It is, therefore, only natural that they should have received considerable attention from food bacteriologists. Winter, Stewart and co-workers (1, 2, 3) have made a series of studies on the extent of contamination to be found in laboratory and commercially prepared liquid egg products, both in the egg yolk and egg white and in mixtures of the two. They also made detailed studies on the types of contaminating organisms present and on the possibility of eliminating some of the undesirable contaminants, such as *Escherichia coli* and *Salmonella* by

pasteurization. Pasteurization at temperatures up to 51°C. for periods of time varying from a few minutes to 20 min. proved effective. This treatment would not bring about coagulation of the albumin nor seriously interfere with the use of egg products in baking and in other uses. Winter (4) investigated a condition called "black rot" in fresh shell eggs, isolated the causative organisms, and indicated ways and means of reducing this type of spoilage. Winter & Wilkin (5) made a study of the effect of freezing, holding, and storage of liquid egg products in order to control the bacterial growth. They reported that during storage in the frozen condition the bacterial count usually decreased. The decrease was most rapid at temperatures close to −18°C.

Solowey, Sutton & Calesnick (6) made a study of the effect of pasteurization on the *Salmonella* found in sprayed dried whole egg powder. Most of these organisms were destroyed in one minute at 58°C. and the most heat resistant ones in 1.2 min. at 61°C. Solowey & Calesnick (7) made a study of *Salmonella* contamination in reconstituted egg powder and showed that the organisms would multiply rapidly after the eggs had been reconstituted. When the egg powder is reconstituted and used for scrambling, most of the *Salmonella* are eliminated but will survive scrambling temperatures of 56°C. for 17 min.

McFarlane *et al.* (8 to 11) and Watson (12) have published a series of papers dealing with the microbiology of sprayed dried whole egg. In these papers they report on the analysis of several thousand samples of sprayed dried whole egg powder received from various manufacturing plants in the United States from the period September 1, 1943 to January 1, 1945. All samples represented lots of powder, manufactured from unpasteurized, liquid whole eggs according to the United States Department of Agricultural and War Food Administration purchase specifications. They were examined by plate and direct microscopic counts, and for *Salmonella* species, *E. coli*, molds, and thermophiles. The monthly average plate count varied from 80,000 to one million organisms per gram. The direct count varied from below ten million to four billion per gram. Egg powder produced from fresh shell egg liquid had a lower count than did the egg powder produced from frozen egg or storage egg liquid. *Salmonella* species were isolated from 35 per cent of the 5,000 samples examined and *E. coli* from 51 per cent. *Salmonella* organisms were found in many samples that gave neg-

ative results for *E. coli* and likewise *E. coli* was found in many samples from which *Salmonella* could not be isolated. About 36 per cent of the egg powder samples had mold counts of 100 or more per gm. and less than 2 per cent had counts of 1,000 or more per gm. Several aerobic sporeformers were found that would grow at 55°C. but these were not strictly thermophilic organisms—strict thermophiles were encountered very infrequently. Similar studies have been made by Hirschamann & Lightbody (13). Cantor & McFarlane (14) report on the occurrence of *Salmonella* organisms in and on chicken eggs and state that the occurrence of these organisms in eggs correlates with the amount of dirt found on the eggs. Solowey (15) and McFarlane & Calesnick (16) published further notes concerning the microbiology of egg powder. Wilson (17) made a study of the occurrence of *Salmonella* organisms in stored egg powder. He studied the survival of the organisms in egg powder that has been stored at temperatures varying from −24° up to 45°C. and reports that if powder is stored at 45°C. it becomes free from *Salmonella* in from 30 to 40 weeks of storage. Similar findings are reported in a special bulletin from the Medical Research Council of Great Britain (18).

Australian workers (19) report the development of a method of pasteurizing whole eggs so as to free them from surface contamination. This is done by passing eggs rapidly through a water bath that is kept at 63.5°C. Stewart & Ayres (20) report that in order to produce a stable egg white powder the sugar should be removed. This can be done by inoculating the egg whites with yeast cells which will remove the sugar completely in three to seven days.

Heat Resistance of Spores

During the past two years a considerable amount of attention has been given to a study of the heat resistance of spores and the ease with which spores will germinate following heat treatment. Stumbo (21) reports on the development of a special apparatus for the study of the heat resistance of spores that will make possible a large series of studies with a saving of time and will also permit the use of higher temperatures, with the correspondingly shorter exposure times, than is possible with conventional techniques. Using high temperatures up to 132°C. he found a straight line relationship to exist when the logarithm of death time was plotted against the temperature.

Curran & Evans (22) and Curran, Evans & Bell (23) report on the effect of heat shock upon the rate of germination and upon the viability of spores following heat shock. They believe that following heat shock, the spores are stimulated to intense metabolic activity. The authors suggest a two-stage method of sterilization; the first being a heat shock, followed by an incubation at 37° to 66°C. for a period not to exceed 5 hr., and then a final pasteurization treatment. Davis & Williams (24) and Davis, Williams & Wyss (25) claim that bacterial spores with a high heat resistance can be developed by selecting those which survive a moderate heat treatment. They also find that the most heat resistant spores have the greatest resistance to disinfectants.

Wynne & Foster (26 to 29) published a series of articles on the physiology of spore germination with special reference to *Clostridium botulinum*. They believe that dormancy is a property of the medium in which the spores are suspended rather than a property of the spores themselves. Some of the factors which may prolong dormancy are the absence of carbon dioxide and the presence of certain types of fatty acids, particularly oleic or linoleic acids; but they also believe that there are other substances, not yet identified, that can prevent spores from germinating for relatively long periods of time.

Gross *et al.* (30 to 36) have published a series of papers on bacteriological studies relating to thermal processing of canned meats. They have used a special technique in which meat paste is processed in standard thermal death time tubes. With this technique they have investigated the heat characteristics of a putrefactive anaerobe which is commonly used as an indicator organism in process evaluations. Thermal death time curves follow the monomolecular law. However, the thermal resistance of this organism, as well as other sporeformers which have been isolated from meats, is influenced by the nature of the medium. If the organisms are grown in raw meat, the thermal resistance may be low; whereas if cooked meat is used, the heat resistance may be very high. In a separate paper Gross & Vinton (37) made a study of the thermal death time of various strains of staphylococci in meat. Here again they have found that the heat resistance can be influenced by the medium in which the organisms have grown. In pasteurized meat some strains survived 100 min. at 66°C. while in sterilized meat the heat resistance found was 13 min. at 66°C. Knaysi (38) published an extensive review on the cytology,

biological nature, chemical composition, and the effect of the environment on the germination of spores. Evans & Curran (39) report that isobutyl vanilate is an effective inhibitor for spore-forming bacteria.

Plant Sanitation

Wolford & Berry (40, 41) made a study of the source of contamination in a plant making orange juice and found that high bacterial counts are common in the slime, wash water, and debris accumulating on various pieces of equipment. In some of this material large numbers of coliform organisms may be present. They also observed that contamination was extensive if the oranges themselves were not carefully selected so as to avoid oranges that were partly spoiled from "soft rot." If unsound oranges are excluded and if the plant is kept perfectly clean, orange juice can be produced with a very low bacterial count. Teunisson & Hall (42) made a study of the bacterial flora of citrus juice and found that most of the organisms isolated can not survive for any long period of time in the juice produced. They also claimed that none of the bacteria produced any noticeable changes in quality, color, or flavor.

Sognefest & Jackson (43) developed a method of sterilizing tomato juice before it is canned by continuously running it through a tubular heat exchanger under pressure. The juice is heated to 121°C. and held for 0.7 of a minute or heated to 138°C. and cooled immediately. A good quality tomato juice with an excellent keeping quality was produced even though it was inoculated beforehand with heat resistant organisms. Wildman & Clark (44) found that *Oidium lactis* can be a common contaminant in tomato juice where slimes are allowed to accumulate on the machinery. Smith (46) made a study of the mold count in tomato products.

Howard & Pederson (45) report that the growth of naturally occurring contaminating bacteria in maple sap causes an increase in alkalinity which in turn may be responsible for a darkening of the color of the resulting maple syrup. To obtain light colored syrup, it is important to maintain equipment in a sanitary condition and to handle the sap rapidly so as not to allow excessive bacterial growth.

Anderson (47) recommends the use of a direct microscopic count as a control of sanitation in a dairy plant, suggesting 200,000 organisms per cc. as the upper limit to be tolerated. Stumbo (48)

stresses the importance of not keeping food at an elevated temperature any longer than the lag phase of the growth curve of the contaminating bacteria.

Niven (49) discusses the effect of bacterial contamination on sausage discolorations and suggests preventive measures. Olson (50) discusses the type of organisms that are apt to be found as contaminants in food plants, depending upon the sources of contamination and the environmental factors. Nagy (51) and Ohart (52) report on experiences with ultraviolet light when used as as aid in sanitation. Nagy finds that colored mold spores have such a high resistance to ultraviolet light that it is impractical to depend on ultraviolet for the destruction of molds in food manufacturing plants. Baren (53) emphasizes that good sanitation can be obtained only when this responsibility is put in the hands of a separate department that is responsible directly to top executives. Feiner (54) reports on the development of a new type of kettle to be used in food manufacturing plants that can be more easily cleaned and kept free from contamination. Lipske & Hubbard (55) discuss the sources of thermophilic contamination in a canning plant and point out how it can be avoided. The authors emphasize that the important thing is to keep the temperature in the plant either below or above a temperature at which the thermophiles can grow, the optimum of which is around 54°C. The best method of reducing the thermophilic count, according to them, is to flood the machinery continuously for 24 hr. with clean water while the machinery is in motion so as to wash out the thermophilic contaminants. Gunderson, Rose & Henn (56) and Gunderson (57, 58) made a thorough study of a chicken boning plant to find out where contamination occurs. They call attention to the very heavy contamination that can result in a plant handling this type of product. Counts of several million per gram of meat were frequently encountered. A number of articles have appeared in the literature giving the results of bacteriological surveys in various food manufacturing plants; thus Bohrer (59) reports on such a survey in a corn canning plant. Vaughn, Winter & Smith (60) made such studies in a plant handling dried fruit.

Frozen Foods

Pederson (61) made a study of the extent of contamination encountered in frozen vegetables and on the effect of various

methods of handling upon this contamination. The greatest danger is to allow the vegetables to become contaminated by the organisms which are in the active growth phase and to hold the food at a temperature at which they continue to grow for any length of time. He states that counts of 10,000 to 100,000 per gram may be expected in frozen vegetables that have been properly handled, but if the counts go above a million per gram, it is an indication of careless handling.

Perry *et al.* (62) have concerned themselves with the danger of *C. botulinum* in frozen vegetables. They have found that none of the samples studied showed any production of botulinum toxin even though many of the samples had been inoculated with these organisms before freezing. *C. botulinum* was isolated both from the inoculated samples and uninoculated controls. The authors believe that the mixed flora which is present in frozen vegetables is responsible for the absence of toxin production. Fitzgerald (63) and Proctor & Nickerson (64) discuss the factors which must be watched in order to produce frozen foods with a reasonably low bacterial count. Fitzgerald believes that frozen foods should not contain more than 100 *E. coli* cells per 100 gm. and that the total plate count should not exceed 100,000 per gm. Proctor & Nickerson believe that a direct microscopic count should be used as a control on the operations in plants manufacturing frozen foods. Gunderson & Rose (65) made a study of the survival of bacteria in precooked fresh frozen foods and report that the population of pathogenic enteric bacilli falls rapidly in chicken chow mein stored at −26°C. during the first 5 days. After that, the rate of death decreases until a more or less resistant population remains. They conclude that cold storage cannot be depended upon to pasteurize frozen foods.

Fish Products

Aschehoug & Vesterhus (66) report on the type of contamination that may be found in winter herring. Strains of *Pseudomonas* are most common in the fresh fish, while *Achromobacter* are more common in stored fish. Castell (67) has made a survey of the extent of contamination in fish filets, the keeping quality of the filets being correlated directly with the extent of contamination. Again Castell (68) has published the first in a series of articles to deal with the various phases of the fishing industry from

the viewpoint of the bacteriologist. Castell & Anderson (69) and Castell (70) made a survey of the presence of anaerobic spore-formers in fish products. They find that such organisms are not encountered frequently and believe that this is due to high salt content.

Germicides and Sanitizing Agents in Food Plants

There is a great deal of literature on the subject of new sanitizing agents and germicides in regard to their effect on sanitation in food plants. Only a few of these articles are covered, those which deal specifically with the effect of these compounds on the sanitary conditions of a plant.

Berstein & Epstein (71) report that they improved the quality of pickles by soaking them in a solution containing a germicidal detergent. This reduced the bacterial count, and furthermore, made the count of the pickles more uniform so that a standard process could be developed that gave consistently good results. A quaternary ammonium compound was found to be the best for the purpose. Mallmann & Zaikowski (72) report good results with the use of a quaternary ammonium compound along with heat in the rinse water in a mechanical dishwashing machine. Johns (73) reports on a glass slide technique for assessing the sanitizing efficiency of quaternary ammonium compounds and hypochlorites. Wolford & Anderson (74) find that propionates are valuable in controlling the growth of microorganisms in fruits and vegetables while they are being prepared for freezing, or while they are in storage prior to preparation for freezing. Penniston & Hedrick (75) were able to reduce the bacterial count substantially in egg products by washing the eggs in a solution containing chlorine or a quaternary ammonium compound. Kivela, Mallmann & Churchill (76) made a study of the mode of action of the surface active agents on spores and vegetative cells. They found that the surface active agents affected the surface primarily and that in many cases the removal of the agent would restore the cells to a viable condition. In many cases where surface active cations are used the spores are actually not killed but simply prevented from germinating. However, vegetative cells could not be revived by removing the germicide.

To prevent contamination in fish it has been advocated that silver be used for the sterilization of sea water. Castell, Ellis &

Anderson (77) show that sodium chloride removed enough of the silver ions to disrupt the germicidal action. In a solution containing 2.5 per cent sodium chloride there was little if any effect of the silver on the bacterial flora.

Food Processing Methods

Stumbo (78), from a theoretical consideration, attempts to ascertain the location in a food container where the probability of bacterial survival is greatest during heat processing. He reaches the rather disturbing conclusion that the center of the can may not be the place where this is most likely to happen. The reviewer is of the opinion, however, that Stumbo's basic assumption needs to be re-examined. Cass (79) has suggested a modification of the Schultz and Olson lethal rate paper for calculating thermal processes for food products in tin containers. Ball (80) has published a series of articles dealing with theoretical considerations concerned with food processing. Martin (81) reports the development of new equipment which allows for the sterilization of fluid products before they are introduced into cans. The process has been successfully carried through a pilot plant stage, and it is claimed that better preservation and high quality can be obtained by the use of this equipment. A sterile, good quality product was produced even when the raw product, the cans, the covers, and the sealing head of the closing machine were heavily inoculated with heat resistant spores.

Merrill (82) had derived constants which may be used for computing sterilization processing times for glass containers. Cathcart, Parker & Beattie (83) and Bartholomew, Harris & Sussex (84) show that loaves of Boston Brown Bread can be treated so as to prevent mold growth by heating with induced current or by the dielectric method. With the dielectric heating a temperature of 150°F. was reached, while with the induced current a surface temperature of 150°F. was attained. Jackson (85) reviews our present state of knowledge concerning the applicability of high frequency electronics for the sterilization of foods. He concludes that the art has not developed as yet to a point where it is practicable. Mickolson (86) claims to have developed equipment that will allow the effective pasteurization of milk with ultraviolet light. The milk is delivered in front of the light in thin films on rotating cylinders.

Microorganisms Responsible for Food Poisoning

Dack (87) states that *C. botulium* and streptococci failed to grow in dried meat with a moisture content less than 30 per cent. *Salmonella* failed to grow with a moisture content less than 50 per cent. Scott & Stewart (88) claimed that *C. botulinum* will grow more readily in canned vegetables in which the cans are lacquered than in those which contain no lacquer. In unlacquered cans, it is claimed enough tin is dissolved to inhibit growth. Anderson & Berry (89) report that certain naturally occurring flavonols found in asparagus inhibit the growth of *C. botulinum*. The most effective compound was quercitrin, which was effective in concentrations of from 80 to 160 p.p.m. Cathcart, Godkin & Barnet (90) made a study of the ability of *Staphylococcus aureus* to grow in various pastry fillings. Chapman (91) reported on a specially improved Stone medium for the isolation and identification of food poisoning staphylococci. Smith & Iba (92) studied the effect of contamination of staphylococci on nut meats and report that at 37°C., or at room temperature, the organisms increase in numbers for several days. This is followed by a drop in population. If the nuts are stored in a refrigerator, the population does not rise but decreases gradually over a long period of time.

Miscellaneous Items

Pederson & Fischer (93) find that there is an antibiotic substance in cabbage tissues that is effective against gram negative bacteria. Hemfeld (94) reports the presence of an antibiotic substance in wheat bran related to a fatty acid which is effective against gram positive organisms, but not against the gram negative bacteria.

Heath (95) made a study of spoilage of dehydrated foods and reports that bacteria are inhibited at a moisture content below 18 per cent, while yeast requires 20 per cent or more, but molds will multiply at 13 per cent moisture.

Ostrolenk *et al.* (96) developed a special medium for the isolation of fecal streptococci which involves incubation at 45°C. in the presence of 0.05 per cent sodium azide. They believe the enterococci are better indices of pollution than *E. coli*. Jensen (97) found that mustiness in foods may be due to the growth of certain varieties of microorganisms. Pederson & Breed (98) have made a study of the fermentation process that goes on in the preparation

of the coffee bean from the harvested berry clusters. The process is a typical lactic acid fermentation such as occurs in other vegetable materials. Rice, Squire & Fried (99) find that the destruction of vitamins in pork may be due to the growth of microorganisms. Epstein (100) reports on a method of testing commercial filter pads that are used to remove microorganisms from liquids in industries. A suspension of *E. coli* is passed through the filters and the filtrate is tested for sterility. Niven (101) has reported the isolation of various types of organisms that can cause green discoloration of sausage. Two organisms belonging to the genera *Lactobacillus* and *Leuconostoc* were particularly active in producing this discoloration. Burrell (102) started publishing a series of articles on various bacteriological problems involved in the manufacture of acid-preserved pickles. Ulrich & Larson (103) have developed a special formula for an indicator to be used in anaerobic jars to determine whether or not anaerobic conditions have been attained.

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THE GROWTH OF BACTERIAL CULTURES

By Jacques Monod
Pasteur Institute, Paris, France

INTRODUCTION

The study of the growth of bacterial cultures does not constitute a specialized subject or branch of research: it is the basic method of Microbiology. It would be a foolish enterprise, and doomed to failure, to attempt reviewing briefly a "subject" which covers actually our whole discipline. Unless, of course, we considered the formal laws of growth for their own sake, an approach which has repeatedly proved sterile. In the present review we shall consider bacterial growth as a method for the study of bacterial physiology and biochemistry. More precisely, we shall concern ourselves with the quantitative aspects of the method, with the interpretation of quantitative data referring to bacterial growth. Furthermore, we shall consider exclusively the positive phases of growth, since the study of bacterial "death," i.e., of the negative phases of growth, involves distinct problems and methods. The discussion will be limited to populations considered genetically homogeneous. The problems of mutation and selection in growing cultures have been excellently dealt with in recent review articles by Delbrück (1) and Luria (2).

No attempt is made at reviewing the literature on a subject which, as we have just seen, is not really a subject at all. The papers and results quoted have been selected as illustrations of the points discussed.

DEFINITION OF GROWTH PHASES AND GROWTH CONSTANTS

Division Rate and Growth Rate

In all that follows, we shall define "cell concentration" as the number of individual cells per unit volume of a culture and "bacterial density" as the dry weight of cells per unit volume of a culture.

Consider a unit volume of a growing culture containing at time t_1 a certain number x_1 of cells. After a certain time has elapsed,

all the cells have divided once. The number of cells per unit volume (cell concentration) is then

$$x = x_1 \cdot 2;$$

after n divisions it will be

$$x = x_1 \cdot 2^n.$$

If r is the number of divisions per unit time, we have at time t_2:

$$x_2 = x_1 \cdot 2^{r(t_2 - t_1)}$$

or using logarithms to the base 2.

$$r = \frac{\log_2 x_2 - \log_2 x_1}{t_2 - t_1} \quad \ldots\ldots\ldots\ldots\ldots [1]$$

where r is the mean division rate in the time interval $t_2 - t_1$. In defining it we have considered the increase in cell concentration. When the average size of the cells does not change in the time interval considered, the increase in "bacterial density" is proportional to the increase in cell concentration. Whether growth is estimated in terms of one or the other variable, the growth rate is the same[1].

However, as established in particular by the classical studies of Henrici (3), the average size of the cells may vary considerably from one phase to another of a growth cycle. It follows that the two variables, cell concentration and bacterial density, are not equivalent. Much confusion has been created because this important distinction has been frequently overlooked. Actually, one or the other variable may be more significant, depending on the type of problem considered. In most of the experimental problems of bacterial chemistry, metabolism, and nutrition, the significant variable is bacterial density. Cell concentration is essential only in problems where division is actually concerned, or where a knowledge of the elementary composition of the populations is important (mutation, selection, etc.).

[1] The use of log base 2 in place of log base 10 simplifies all the calculations connected with growth rates. It is especially convenient for the graphical representation of growth curves. If $\log_2$ of the bacterial density ($\log_2 = 3.322 \log_{10}$) is plotted against time, an increase of one unit in ordinates corresponds to one division (or doubling). The number of divisions that have occurred during any time interval is given by the difference of the ordinates of the corresponding points. It is desirable that this practice should become generalized.

Although the two variables are not equivalent, it is convenient to express growth rates in the same units (i.e., number of doublings per hour) in both cases. When cell concentrations have been estimated, it is equivalent to the true division rate. When bacterial density is considered, it expresses the number of doublings of bacterial density per unit time, or the division rate of cells postulated to be of constant average size. In all that follows, unless specified, we shall consider growth and growth rates in terms of bacterial density.

These definitions involve the implicit assumption that in a growing culture all the bacteria are viable, i.e., capable of division or at least that only an insignificant fraction of the cells are not capable of giving rise to a clone. This appears to be a fairly good assumption, provided homogeneous populations only are considered. It has been challenged however [Wilson (4)] on the basis of comparisons of total and viable counts. But the cultures examined by Wilson were probably not homogeneous (see p. 378), and the value of the viable count in determining the "absolute" number of cells which should be considered viable under the conditions of the culture is necessarily doubtful (see p. 378). Direct observations by Kelly & Rahn (5) contradict these findings and justify the assumption. [See also Lemon (42) and Topley & Wilson (43).]

Growth Phases

In the growth of a bacterial culture, a succession of phases, characterized by variations of the growth rate, may be conveniently distinguished. This is a classical conception, but the different phases have not always been defined in the same way. The following definitions illustrated in Fig. 1 will be adopted here:

1. lag phase: growth rate null;
2. acceleration phase: growth rate increases;
3. exponential phase: growth rate constant;
4. retardation phase: growth rate decreases;
5. stationary phase: growth rate null;
6. phase of decline: growth rate negative.

This is a generalized and rather composite picture of the growth of a bacterial culture. Actually, any one or several of these phases may be absent. Under suitable conditions, the lag and acceleration

phases may often be suppressed (see p. 388). The retardation phase is frequently so short as to be imperceptible. The same is sometimes true of the stationary phase. Conversely, more complex growth cycles are not infrequently observed (see p. 389).

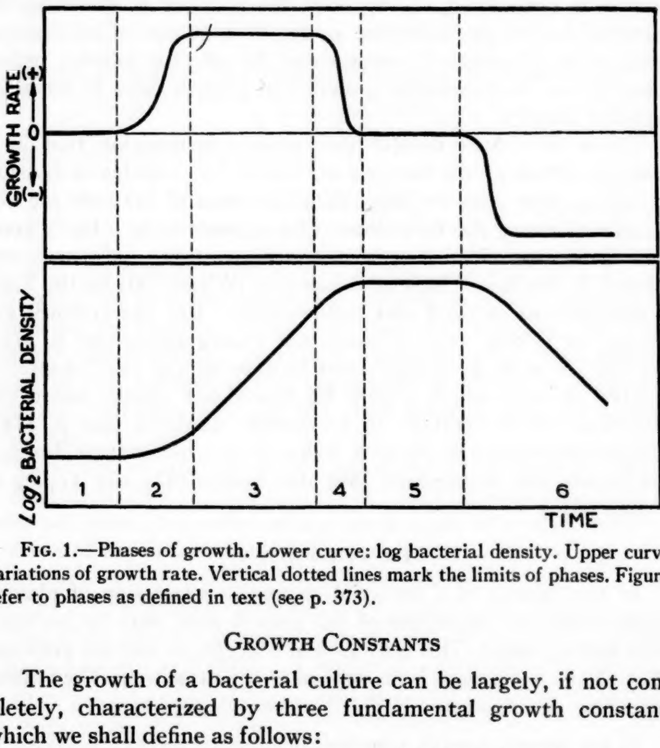

FIG. 1.—Phases of growth. Lower curve: log bacterial density. Upper curve: variations of growth rate. Vertical dotted lines mark the limits of phases. Figures refer to phases as defined in text (see p. 373).

GROWTH CONSTANTS

The growth of a bacterial culture can be largely, if not completely, characterized by three fundamental growth constants which we shall define as follows:

Total growth:[2] difference between initial (x_0) and maximum (x_{max}) bacterial density:

$$G = x_{max} - x_0.$$

Exponential growth rate: growth rate during the exponential phase (R). It is given by the expression

$$R = \frac{\log_2 x_2 - \log_2 x_2}{t_2 - t_1}$$

[2] "Croissance totale," Monod 1941.

when $t_2 - t_1$ is any time interval within the exponential phase.

Lag time and growth lag.—The lag is often defined as the duration of the lag phase proper. This definition is unsatisfactory for two reasons: (*a*) it does not take into account the duration of the acceleration phase; (*b*) due to the shape of the growth curve, it is difficult to determine the end of the lag phase with any precision.

As proposed by Lodge & Hinshelwood (6), a convenient lag constant which we shall call lag time (T_l) may be defined as the difference between the observed time (t_r) when the culture reaches a certain density (x_r) chosen within the exponential phase, and the "ideal" time at which the same density would have been reached (t_i) had the exponential growth rate prevailed from the start, i.e., had the culture grown without any lag $T_l = t_r - t_i$, or

$$T_l = t_r - \frac{\log_2 x_r - \log_2 x_0}{R}.$$

The constant thus defined is significant only when cultures having the same exponential growth rate are compared. A more general definition of a lag constant should be based on physiological rather than on absolute times. For this purpose, another constant which may be called growth lag (L) can be defined as

$$L = T_l \cdot R.$$

L is the difference in number of divisions between observed and ideal growth during the exponential phase. T_l and L values are conveniently determined graphically (Fig. 2).

ON TECHNIQUES

Estimation of Growth

Bearing these definitions in mind, a few general remarks may be made about the techniques employed for the estimation of bacterial density and cell concentrations.

Bacterial density.—For the estimation of bacterial density, the basic method is, by definition, the determination of the dry weights. However, as it is much too cumbersome (and accurate only if relatively large amounts of cells can be used) it is employed mainly as a check of other indirect methods.

Various indirect chemical methods have been used. Nitrogen determinations are generally found to check satisfactorily with

dry weights. When cultures are grown on media containing an ammonium salt as sole source of nitrogen, estimations of the decrease of free ammonia in the medium appear to give adequate results (7). Estimations of metabolic activity (oxygen consumption, acid production) may be convenient (8), but their use is obviously

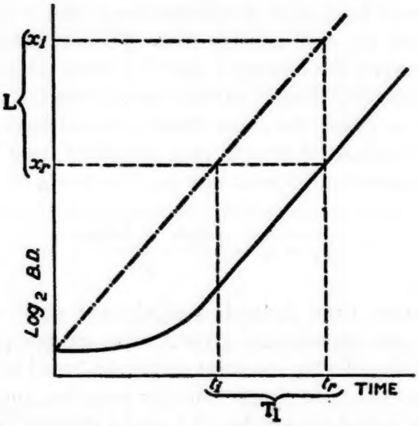

FIG. 2.—Lag time and growth lag. Solid line = observed growth. Dotted line = "ideal growth" (without lag). T_l = lag time. L = growth lag. (See text p. 375.)

very limited. Centrifugal techniques have been found of value (9).

The most widely used methods, by far, are based on determinations of transmitted or scattered light. (Actually, the introduction around 1935 of instruments fitted with photoelectric cells has contributed to a very large degree to the development of quantitative studies of bacterial growth.) We cannot go here into the physical aspects of this problem [for a discussion of these see (10)]. What should be noted is that in spite of the widespread use of the optical techniques, not enough efforts have been made to check them against direct estimations of cell concentrations or bacterial densities. Furthermore a variety of instruments, based on different principles, are in use. The readings of these instruments are often quoted without reference to direct estimations as arbitrary units of turbidity, the word being used in an undefined

sense, or as "galvanometer deflections" which is worse. This practice introduces no little confusion and indeterminacy in the interpretation and comparison of data. It should be avoided.

Whatever instruments are used, the readings should be checked against bacterial density or cell concentration determinations, and the checks should be performed not only on different dilutions of a bacterial suspension, but at various times during the growth of a control culture. Only thus will the effects of variations of size of the cells be controlled. Without such controls it is impossible to decide whether the readings can be interpreted in terms of bacterial density or cell concentration, or both, or neither.

Actually, the instruments best fitted for the purpose appear to be those which give readings in terms of optical density (log I_0/I). With cultures well dispersed, it is generally found that optical density remains proportional to bacterial density throughout the positive phases of growth of the cultures (11). When this requirement is fulfilled, optical density determinations provide an adequate and extremely convenient method of estimating bacterial density.

It is often convenient to express optical density measurements in terms of cell concentrations. For this purpose, the two estimations should be compared during the exponential phase. The data, expressed as cell concentrations, may then be considered as referring to "standard cells," equal in size to the real bacteria observed during the exponential phase, larger than bacteria in the stationary phase and probably smaller than those in the acceleration phase.

Cell concentration.—Cell concentration determinations are performed either by direct counts (total counts) or by indirect (viable) counts. The value of the first method depends very much on technical details which cannot be discussed here. Its interpretation depends on the properties of the strains (and media) and is unequivocal only with organisms which do not tend to remain associated in chains or clumps. Total counts are evidently meaningless when there is even a slight tendency to clumping.

The same remarks apply to the indirect, so called viable, counts made by plating out suitable dilutions of the culture on solid media. The method has an additional difficulty, as it gives only the number of cells capable of giving rise to a colony on agar under conditions widely different from those prevailing in the culture. Many organisms, such as pneumococci (12), are extremely sensitive to

sudden changes in the composition of the medium. The mere absence of a carbon source will induce "flash lysis" of *Bacillus subtilis* (13). Such effects may be, in part at least, responsible for the discrepancies often found between total and viable counts.

In spite of these difficulties viable counts retain the undisputed privilege of being by far the most sensitive method and of alone permitting differential counting in the analysis of complex populations. In the latter case, relative numbers are generally the significant variable, and whether or not the counts give a reasonably accurate estimation of absolute cell concentrations is unimportant.

Methods of Culture

Although the methods of culture will vary according to the problems investigated, certain general requirements must be met in any case. The most important one is that the cultures should be constantly mixed, homogenized, and in equilibrium with the gas phase. This is achieved either by shaking or by bubbling air (or other gas mixtures) or both. Bubbling is often found inefficient unless very vigorous, when it may provoke foaming which should be avoided. Slow rocking of a thin layer of liquid is the simplest and probably the best procedure. [For detailed descriptions of techniques, see (14).]

Various techniques for the continuous renewal of the medium have been described (15) and should be found useful for certain types of experiments (see p. 385).

The composition of the medium is largely determined by the nature of the experiment, and the properties of the strains. One general rule should however, so far as possible, be followed in the planning of quantitative growth experiments. As a culture grows, the conditions in the medium alter in a largely uncontrollable and unknown way. Therefore, the observations should be performed while the departure from initial conditions may still be considered insignificant. The more dilute the cultures, the closer will this requirement be met. The sensitivity of optical density measurements makes it practicable to restrict most experiments to a range of bacterial densities not exceeding 0.25 mg. dry weight per ml.

THE PHYSIOLOGICAL SIGNIFICANCE OF THE GROWTH CONSTANT

Total Growth

Limiting factors.—The metabolic activity of bacterial cells

modifies the composition of the medium in which they grow. Depending on the initial conditions, and on the properties of the strains, one or another, or several, of these changes will eventually result in a decrease of the growth rate, bringing the exponential phase to an end, and leading more or less rapidly to the complete cessation of growth.

The factors most commonly found to be limiting can, as a rule, be classified in one of the following groups: (*a*) exhaustion of nutrients; (*b*) accumulation of toxic metabolic products; and (*c*) changes in ion equilibrium, especially pH.

The physiological significance of the constant *G* (total growth) depends on the nature of the limiting factor. It is uninterpretable when the limiting factor is unknown, or when several factors cooperate in limiting growth. The conditions of an experiment where *G* is to be estimated must therefore be such that a single limiting factor is at work. This may be considered to be the case only where it can be shown that no change, other than the one considered, plays a significant part both in breaking the exponential phase and in stopping the growth. Provided these requirements are met, the utilization of *G* as a measure of the effect of a limiting factor is warranted.

Actually, the estimation of *G* is especially useful when the limiting factor is a single, known, essential nutrient. Under such conditions, it can be a most convenient tool for the study of many aspects of nutritional problems. The principles of this technique and some general results will be considered in the next paragraphs.

Nutrients as limiting factors.—The bacteria most commonly studied are chemoorganotrophs[3] requiring an organic compound as carbon and energy source, a hydrogen acceptor, inorganic ions, and carbon dioxide. Most of the parasitic (and many saprophytic) bacteria are chemoorganoheterotrophs requiring, in addition to the above diet, certain specific organic molecules (growth factors).

Any one of the essential nutritional requirements of an organism is, by definition, a potential limiting factor. With organisms able to grow on simple defined media (whether they are organoautotrophs or organoheterotrophs), the composition of a medium is easily adjusted so that the concentrations of all essential nutrients are in large excess compared to one of them, which then becomes the sole limiting factor, provided its concentration is kept

[3] The Cold Spring Harbor Nomenclature (16) is adopted here.

low enough to eliminate interference from other potential limiting factors (pH changes, accumulation of metabolic products, etc.). Within the limits thus defined, the relation between G and the initial concentration (C) of the nutrient is, as a very general rule, found to be the simplest possible, namely, linear and to conform to the equation:

$$G = KC.$$

This relation implies that the amount of limiting nutrient used up in the formation of a unit quantity of cell substance is independent of the concentration of the nutrient. It implies also that growth stops only when the limiting nutrient is completely exhausted, or, in other words, that there is no threshold concentration below which growth is impossible (11).

Neither of these conclusions can be considered strictly true of course, and the linear relation cannot be taken for granted a priori. But it does seem to be a general approximation, and even a remarkably accurate one in many cases (Fig. 3 and Table I). Where

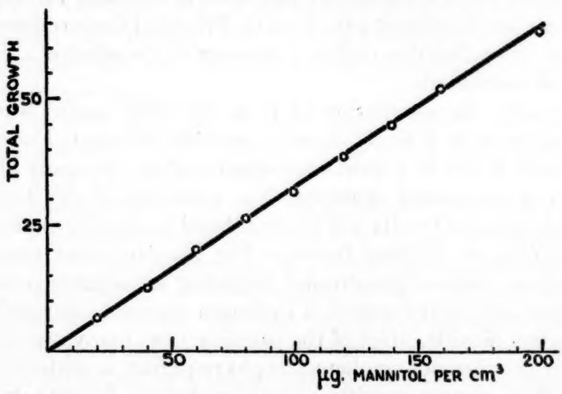

FIG. 3.—Total growth of *E. coli* in synthetic medium with organic source (mannitol) as limiting factor. Ordinates: arbitrary units. One unit is equivalent to 0.8 μg. dry weight per ml. (11).

it holds, the estimation of G affords a simple and direct measure of the growth yield (K) on the limiting nutrient, or

$$\frac{G}{C} = K = \frac{\text{amount of bacterial substance formed}}{\text{amount of limiting nutrient utilized}}.$$

When the proportion of the dry weight representing substance derived from the limiting nutrient is known, it is a measure of the fraction assimilated. If G is expressed as "standard" cell concentration, $1/K$ represents the amount of limiting nutrient used up in the formation of a "standard" cell. Thus, when determined under proper conditions, G is a constant of perfectly clear and fundamental significance; it is a measure of the efficiency of assimilatory processes.

TABLE I

Total growth of purple bacteria with acetate as limiting factor [after Van Niel (9)]

Acetate (mg/ml.)	0.5	1.0	2.0	3.0
Total growth (mg/ml.)	0.18	0.36	0.70	1.12
K	0.36	0.36	0.35	0.37

Extensive data on G and K values are available only with respect to the organic source (9, 11). Little is known of K values in the case of inorganic sources. Owing to the development of microbiological assay methods, abundant data are available on the quantitative relations between growth of many bacteria and concentration of a variety of growth factors. But the major part of these data do not bear any known relation to G or any other definable growth constant, which is most unfortunate. It does seem at least probable that in many instances, the measurement of total growth, under conditions insuring homogeneity and limitation of growth by a single factor, could with advantage replace estimations of "turbidity at 16 hours," or "galvanometer deflections at 24 hours." It can be predicted with confidence that in most cases linear relations would be found [see e.g. (44)], permitting the estimation of K, and on which simpler and more reproducible methods of assay could be based. Furthermore, an intelligible and very valuable body of quantitative data on nutritional requirements of bacteria would thus become accumulated.

The remarkable degree of stability and reproducibility of K values, for a given strain and a given compound under similar

conditions, should be emphasized. Contrary to the other growth constants, it seems to be very little affected by hereditary variability (45).

In general, of the three main growth constants, total growth is the easiest to measure with accuracy and the most stable. Its interpretation is simple and straightforward, provided certain experimental requirements are met. These are remarkable properties, which could, it seems, be put to much wider use than has hitherto been done, especially with the focussing of attention on problems of assimilatory and synthetic metabolism.

Exponential Growth Rate

The exponential phase as a steady state: rate determining steps.— The rate of growth of a bacterial culture represents the over-all velocity of the series of reactions by virtue of which cell substance is synthesized. Most, if not all, of these reactions are enzymatic, the majority probably are reversible, at least potentially. The rate of each, considered alone, depends on the concentrations of the reactants (metabolites) and on the amount of the catalyst (enzyme).

During the exponential phase, the growth rate is constant. It is reasonable to consider that a steady state is established, where the relative concentrations of all the metabolites and all the enzymes are constant. It is in fact the only phase of the growth cycle when the properties of the cells may be considered constant and can be described by a numeric value, the exponential growth rate, corresponding to the over-all velocity of the steady state system.

It has often been assumed that the over-all rate of a system of linked reactions may be governed by the slowest, or master, reaction. That this conception should be used, if at all, with extreme caution, has also been emphasized (17, 18). On theoretical grounds, it can be shown that the over-all rate of a system of several consecutive reversible enzymatic reactions depends on the rate and equilibrium constant of each. The reasons for this are obvious, and we need not go into the mathematics of the problem. A master reaction could take control only if its rate were very much slower than that of all the other reactions. Where hundreds, perhaps thousands, of reactions linked in a network rather than as a chain are concerned, as in the growth of bacterial cells, such a

situation is very improbable and, in general, the maximum growth rate should be expected to be controlled by a large number of different rate-determining steps. This makes it clear why exponential growth rate measurements constitute a general and sensitive physiologic test which can be used for the study of a wide variety of effects, while, on the other hand, quantitative interpretations are subject to severe limitations. Even where the condition or agent studied may reasonably be assumed to act primarily on a single rate determining step, the over-all effect (i.e., the growth rate) will generally remain an unknown function of the primary effect.

Although very improbable, it is of course not impossible that the exponential growth rate could in certain specific cases actually

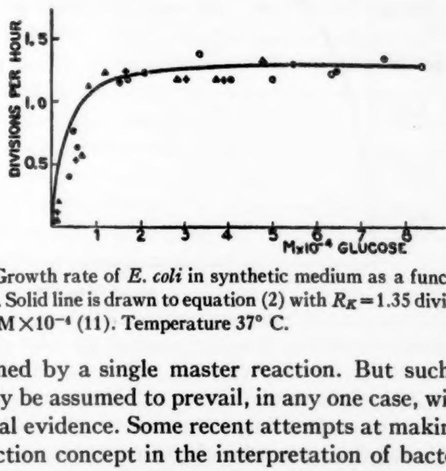

FIG. 4.—Growth rate of *E. coli* in synthetic medium as a function of glucose concentration. Solid line is drawn to equation (2) with $R_K = 1.35$ divisions per hour, and $C_1 = 0.22$ M$\times 10^{-4}$ (11). Temperature 37° C.

be determined by a single master reaction. But such a situation could hardly be assumed to prevail, in any one case, without direct experimental evidence. Some recent attempts at making use of the master reaction concept in the interpretation of bacterial growth rates are quite unconvincing in that respect (19).

Rate-concentration relations.—Notwithstanding these difficulties, relatively simple empirical laws are found to express conveniently the relation between exponential growth rate and concentration of an essential nutrient. Examples are provided in Figs. 4 and 5. Several mathematically different formulations could be made to fit the data. But it is both convenient and logical to adopt a hyperbolic equation:

$$R = R_K \frac{C}{C_1 + C} \qquad [2]$$

similar to an adsorption isotherm or to the Michaelis equation. In the above equation C stands for the concentration of the nutrient. R_K is the rate limit for increasing concentrations of C. C_1 is the concentration of nutrient at which the rate is half the maximum.

The constant R_K is useful in comparing efficiency in a series of related compounds as the source of an essential nutrient. So far extensive data are available only with respect to the organic source (11). The value of R_K may vary widely when different

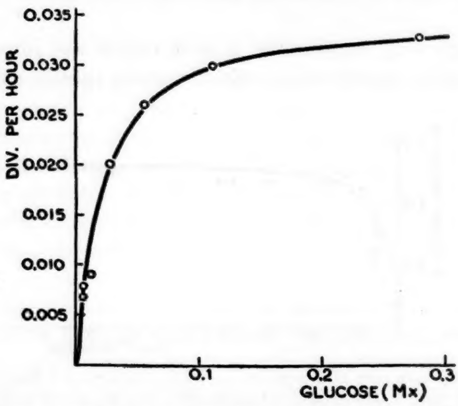

FIG. 5.—Growth rate of *M. tuberculosis* in Dubos' medium, as a function of glucose concentration. Solid line drawn to equation (2) with $R_K = 0.037$ and $C_1 = M/45$ (20).

organic sources are compared under otherwise identical conditions. There is no doubt that it is related to the activity of the specific enzyme systems involved in the breakdown of the different compounds, and it can be used with advantage for the detection of specific changes (e.g., hereditary variation) affecting one or another of these systems (30).

The value of C_1 should similarly be expected to bear some more or less distant relation to the apparent dissociation constant of the enzyme involved in the first step of the breakdown of a given compound. Furthermore, since a change of conditions affecting primarily the velocity of only one rate-determining step will, in general (but not necessarily), be only partially reflected in the

over-all rate, one might expect C_1 values to be lower than the corresponding values of the Michaelis constant of the enzyme catalysing the reaction. This may explain why C_1 is often so small, compared to the concentrations required for visible growth, that its value may be difficult to determine, and the exponential growth rate appears practically independent of C (19).

It may be of interest to note that in a few instances exceptionally large values of C_1 have been obtained. For instance for *Mycobacterium tuberculosis*, on Dubos' medium, the value of C_1 for glucose is M/45, i.e., some 1,000 times its value for *Escherichia coli*. Whether this is due to a very low affinity of an enzyme or whether it reflects a peculiar permeability property of the membrane of this organism is not known (20).

Growth rate determinations as a null point method.—Although the growth rate is an unknown function of a large number of variables, quantitative comparisons of the effects of conditions or agents affecting it through the same rate-determining reaction (or system of reactions) are possible (at least in principle) by using growth rate measurements as a null point method, that is to say by determining the equivalent conditions at which a certain, conveniently chosen, value of R obtains. This general method is susceptible of many applications, especially in the study of antagonistic effects. Here reliable and sensitive methods for distinguishing between various types of antagonistic effects, and determining the relative activities of different antagonists, are needed. Theoretically the most sensitive comparisons should be afforded by determining, at various absolute concentrations, the ratios of inhibitor and antagonist at which a given per cent decrease of R (over uninhibited controls) occurs.

Although this may not always prove practicable, there is little doubt that growth rate measurements do yield data, not only more accurate, but essentially more informative, than "turbidity at 16 hours" or "galvanometer deflections at 72 hours." The studies of McIllwain on the pantoyl taurine-pantothenate antagonism (8) adequately illustrate this point. They clearly show, in particular, the importance of distinguishing between effects on growth rate and on total growth [see also (21 to 24)].

Linear growth.—Since we are discussing the interpretation of exponential growth rates, it may be worthwhile to consider the case when growth is linear with time, although, to the reviewer's

knowledge, this has been clearly observed only once (25), actually during the residual growth of a streptomycin-requiring *B. subtilis* in a medium containing no streptomycin (Fig. 6). The interpretation is obvious, albeit surprising. Growth must be limited by one enzyme or system of enzymes, the activity of which is constant. In other words, in the absence of streptomycin, one rate-determining enzyme ceases to be formed, so that by being outgrown by the

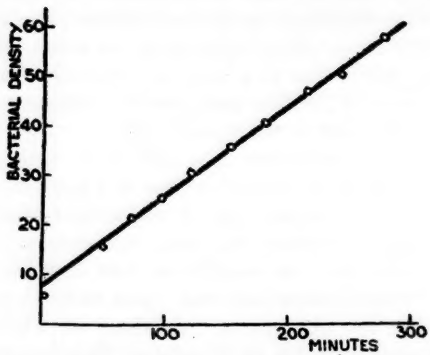

FIG. 6.—Residual growth of a streptomycin requiring strain of *Bacillus subtilis* in the absence of streptomycin. Growth is linear for over 4 hr. (25).

other enzymes, it eventually achieves true mastery and sets the system at its own constant pace, disregarding the most fundamental law of growth.

Similar systems could be artificially set up by establishing a constant, limited supply of an essential metabolite (using an organism incapable of synthesizing it), while all other nutrients would be in excess. Such a technique should prove useful for certain studies of metabolism (see p. 378).

LAG TIME

Types of lag.—The lag and acceleration phases correspond to the gradual building up of a steady state. The growth lag (*L*) may be considered a measure of the physiological distance between the initial and the steady state. Depending on the specific conditions and properties of the organism, one or several or a large number of reactions may determine the rate of this building up

process. Furthermore each rate-determining reaction may be affected in either or both of two ways: (*a*) change in the amount and activity of the catalyst; (*b*) change in the concentration of the reactants (metabolites).

When the phenomenon is associated with the previous ageing of the cells of the inoculum, the chances are that it involves at once a large number of reactions, and specific interpretations are impossible. Furthermore an apparent lag may be caused if a large fraction of the incoulated cells are not viable (18). When, however, the lag can be shown to be controlled primarily by only one reaction, or system of reactions, the measurement of lag times becomes a useful tool for the study of this reaction. This may often be achieved by a careful preconditioning of the inoculated cells, and appropriate choice of media [see e.g. (26)]. In point of fact this technique amounts to artificially creating conditions where one or a few rate limiting steps become true master reactions, at least during the early stages of the lag.

Theoretically, the lagging of a reaction may be due either to insufficient supply of a metabolite or to the state of inactivity of the enzyme. In the first case, the technique may be used for the study of certain essential metabolites synthesized by the cell itself during growth, and consequently difficult to detect and identify otherwise. Few examples of this sort are available besides the glutamine effects studied by McIlwain *et al.* (27) and the detection of metabolites able to replace carbon dioxide (26), but it is probable that the method could be developed.

In the second case, the technique may be useful in the study of enzyme activation or formation. The magnesium effects described by Lodge & Hinshelwood (28) and the sulfhydryl effects described by Morel (29) should probably be attributed to the reactivation of certain enzymes or group of enzymes. However, lag effects are especially interesting in connection with the study of enzymatic adaptation.

Lag and enzymatic adaptation.—Enzymatic adaptation is defined as the formation of a specific enzyme under the influence of its substrate (30). If cells are transferred into a medium containing, as sole source of an essential nutrient, a compound which was not present in the previous medium, growth will be impossible unless and until an enzyme system capable of handling the new substrate is developed. If other potential factors of lag are elimi-

nated, the determination of lag times becomes a means of studying the adaptive properties of the enzyme system involved (Fig. 7).

The technique has proved especially useful for the study of adaptive enzymes attacking organic compounds serving as sole organic source (11, 31). The work of Pollock (32) shows that it can also be applied in the case of adaptive systems specific for certain hydrogen acceptors (nitrate and tetrathionate). A further development of the technique is suggested by the work of Stanier

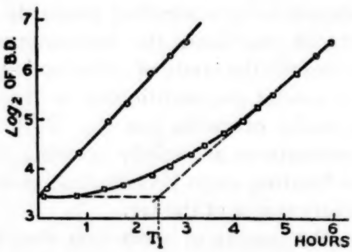

FIG. 7.—Growth of *E. coli* in synthetic medium with glucose (circles) and xylose (squares) as organic source. Culture previously maintained on arabinose medium, temperature 37° C. Growth on glucose proceeds without any lag. Lag time (T_l) on xylose is approximatively 2.5 hours (46).

(33) and Cohen (34) on the possibility of identifying metabolic pathways through a systematic study of cross adaptation.

In general, lag-time measurements may be especially useful in the detection and preliminary identification of adaptive effects, but they could not, of course, replace more direct methods of estimating enzymatic activities.

A broader approach to the problem of relations between lag and enzymatic adaptation should also be considered. As emphasized by Hinshelwood (18), the lag and acceleration phases represent essentially a process of equilibration, the functioning of a regulatory mechanism, by virtue of which a certain enzyme balance inside the cells is attained. That such a mechanism must exist is obvious, since in its absence, the cells could not survive even slight variations of the external environment. However, the nature of the postulated mechanisms is still completely obscure. The kinetic speculations of Hinshelwood, although interesting as empirical formulations of the problem, do not throw any light on

the nature of the basic mechanisms involved in the regulation of enzyme formation by the cells.

The most promising hypothesis for the time being appears to be that this regulation is insured through the same mechanism as the formation of adaptive enzymes, which implies the assumption that all the enzymes in a cell are more or less adaptive. The competitive effects observed in enzymatic adaptation (11, 35, 36) agree with the view that the regulation may be the result of a continuous process of selection of mutually interacting enzymes or enzyme-forming systems (30, 37). The kinetics of bacterial growth and, in particular, the lag and acceleration phases certainly constitute the best available material for the study of this fundamental problem.

Division lag.—The largest discrepancies between increase in bacterial density and increase in cell concentration are generally observed during the lag and acceleration phases. This phenomenon has been the subject of much confused discussion (38). Actually, it has been demonstrated by Hershey (39, 40) that a definite lag in cell concentration may occur even when there is no detectable lag in bacterial density. This must mean that cell division mechanisms may be partially inhibited under conditions which do not affect the growth rate and general metabolism of the cell. A number of interesting observations by Hinshelwood *et al.* (18) point to the same conclusion. Further studies on the phenomenon are desirable, as they should throw some light on the factors of cell division in bacteria.

The Interpretation of Complex Growth Cycles

Multiple exponential phases.—In many cases, the growth cycle does not conform to the conventional scheme represented in Fig. 1. The interpretation of these complex growth cycles will be briefly discussed here.

One of the most frequently encountered exceptions is the presence of several successive exponential phases, characterized by different values of R and separated by angular transition points. This should in general be interpreted as indicating the addition or removal of one or more rate-determining steps in the steady state system. This type of effect may result from a change in the composition of the medium, for instance from the exhaustion of a compound partially covering an essential nutritional requirement

(34), or from the transitory accumulation of a metabolite, which will eventually serve as a secondary nutritional source (41).

Interpretations are more delicate, and more interesting, when the cause is a change in the composition of the cells themselves. Such effects are frequently encountered with various bacteriostatic agents and have been discussed at length by Hinshelwood (18). But the deliberate confusion entertained by this author between selective and adaptive mechanisms has obscured, rather than clarified, the interpretation of these effects.

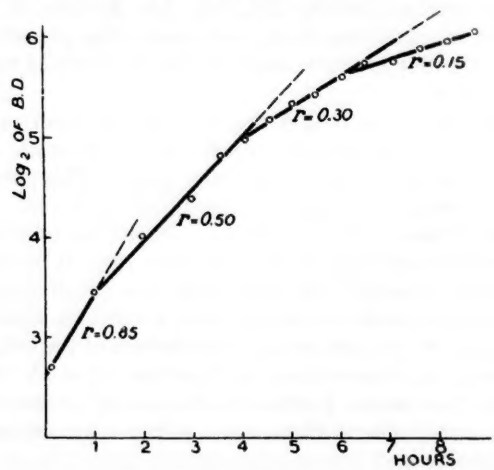

FIG. 8.—Growth of *E. coli* in synthetic medium under suboptimal partial pressure of carbon dioxide (3×10^{-5}). r = growth rate.

In some cases, the phenomenon can be reasonably ascribed to the exhaustion of a reserve metabolite in the cells. An interesting example is afforded by the growth of coli under suboptimal partial pressures of carbon dioxide (26). As seen in Fig. 8 as much as three or four exponential phases can be clearly distinguished suggesting the successive exhaustion of several reserve metabolites, each independently synthesized with the participation of carbon dioxide, a conclusion which is borne out by other lines of evidence.

Diauxie.—This phenomenon is characterized by a double growth cycle consisting of two exponential phases separated by a phase during which the growth rate passes through a minimum,

even becoming negative in some cases. It is found to occur in media where the organic source is the limiting factor and is constituted of certain mixtures of two carbohydrates. The evidence indicates that each cycle corresponds to the exclusive utilization of one of the constituents of the mixture, due to an inhibitory effect of one of the compounds on the formation of the enzyme attacking the other (Fig. 9). This striking phenomenon thus reveals the existence

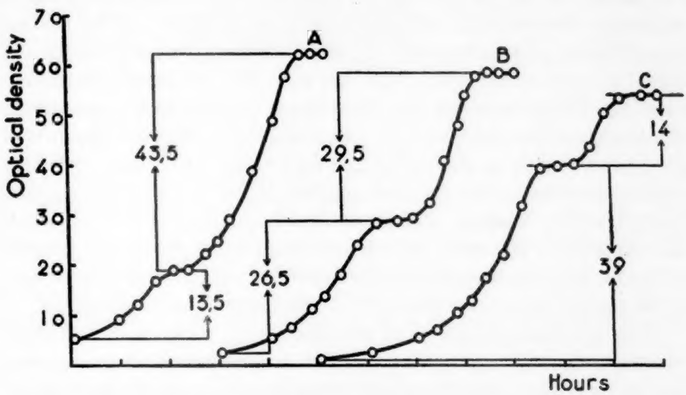

FIG. 9.—Diauxie. Growth of *E. coli* in synthetic medium with glucose+sorbitol as carbon source.

The figures between arrows indicate total growth corresponding to each cycle.

(*a*) Glucose 50 μg. per ml.; sorbitol 150 μg. per ml.
(*b*) Glucose 100 μg. per ml.; sorbitol 100 μg. per ml.
(*c*) Glucose 150 μg. per ml.; sorbitol 50 μg. per ml.

Total growth corresponding to first cycle is proportional to glucose concentration. Total growth of second cycle is proportional to sorbitol concentration (11).

of interactions between closely related compounds in the formation of specific enzymes and has proved valuable in the study of certain aspects of enzymatic adaptations (11, 30, 35). It may perhaps be susceptible of certain technical applications, e.g., for the quantitative analysis of certain mixtures of carbohydrates.

CONCLUDING REMARKS

The time-honored method of looking at a tube, shaking it, and looking again before writing down a + or a 0 in the lab-book has led to many a great discovery. Its gradual replacement by

determinations of "turbidity at 16 hours" testifies to technical progress, primarily in the manufacturing and advertising of photelectric instruments. This technique however is not, properly speaking, quantitative, since the quantity measured is not defined. It might be a rate, or a yield, or a combination of both.

In any case, this technique does not take advantage of the fact that the growth of bacterial cultures, despite the immense complexity of the phenomena to which it testifies, generally obeys relatively simple laws, which make it possible to define certain quantitative characteristics of the growth cycle, essentially the three growth constants: total growth (G), exponential growth rate (R), and growth lag (L). That these definitions are not purely arbitrary and do correspond to physiologically distinct elements of the growth cycle is shown by the fact that, under appropriately chosen conditions, the value of any one of the three constants may change widely without the other two being significantly altered. The accuracy, the ease, the reproducibility of bacterial growth constant determinations is remarkable and probably unparallelled, so far as biological quantitative characteristics are concerned.

The general physiological significance of each of the growth constants is clear, provided certain experimental requirements are met. Under certain specific conditions, quantitative interpretations in terms of the primary effect of the agent studied may even be possible. The fallacy of considering certain naive mechanistic schemes, however, as appropriate interpretations of unknown, complex phenomena should be avoided.

There is little doubt that, as further advances are made towards a more integrated picture of cell physiology, the determination of growth constants should and will have a much greater place in the experimental arsenal of microbiology.

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RECENT DEVELOPMENTS IN MICROBIOLOGICAL TECHNIQUES

By L. S. McClung
Department of Bacteriology, Indiana University
Bloomington, Indiana

Within the limited space available for this review obviously it will not be possible even to list, much less to evaluate, each of the newly devised tools and/or techniques which have appeared in recent years. Therefore, this account will be concerned mainly with recent trends in techniques with an indication of the reference material.

Since a particular technique often is concerned with new apparatus, or with further use of instruments already known, mention will also be made of some of the reference material concerning specialized apparatus currently being used in microbiological laboratories. Although this topic has not been reviewed recently, only publications which have appeared within the last few years will be included and often the references cited are intended to be illustrative and not as a complete bibliography.

Owing to space limitations and the field of interest of the reviewer, this summary will be concerned mainly with the techniques for study of the bacteria and their products, though some reference will be made to the other microorganisms. This review will not contain many references to new or revised procedures for staining microorganisms (these are abstracted in *Stain Technology* and elsewhere) nor formulae of media, specific cultural reactions, or procedures of interest mainly to the clinical or diagnostic laboratories. Material of the latter nature may be found in such books as those of Gradwohl (134), Marshall *et al.* (259), Mackie & McCartney (256), Schaub & Foley (329), Stitt, Clough & Branham (351), Wadsworth (388), or the *Pure Culture Study of Bacteria* (302) issued by the Committee on Technic of the Society of American Bacteriologists. Other recent books which include, or are devoted exclusively to, discussions of techniques relate to viruses (118), histo- and cytochemistry (130), immunochemistry (195), immunology (50), enzymes (358, 366), biochemical preparations (71) and general laboratory techniques (65, 99, 153, 190). Mention

should also be made of the excellent symposia of the New York Academy of Sciences, which recently have included material on the ultracentrifuge (248), chromatography (73), and electrophoresis (249). A welcome response should be accorded the new annual reviews in *Analytical Chemistry*, which, within two months, summarized recent developments concerning electron microscopy (63), light microscopy (191), light absorption spectrometry (267), infrared spectroscopy (22), emission spectroscopy (266), mass spectrometry (170), polarography (237), chromatography (354), statistics (397), and organic microchemistry (410).

The modern microbiologist, depending on his field of interest, may need to be familiar with the recent advances in other areas of biology, physics, chemistry (physical, biological, and organic), genetics, and, in addition, to have a working knowledge of statistics. For those who seek information on new techniques, many references may be found in the section on *Bacteriological Apparatus and Methods* in *Biological Abstracts*, in the new *Excerpta Medica* or *Bulletin Analytique* of the Centre National de la Recherche in Paris or other abstract journals, but, for apparatus, careful attention should be given to announcements in (*a*) *Review of Scientific Instruments;* (*b*) *Instruments;* (*c*) *Instrument Practice;* and (*d*) *Journal of Scientific Instruments*, in addition to the usual research journals.

Enrichment techniques.—The principle and applications of selective enrichment for isolation of antibiotic producing organisms has been covered fully by Waksman (389, 391) and others. Barker and his students have been successful in isolating from nature, by use of selective media, several organisms of value in metabolic studies. These include *Clostridium propionicum*, which produces propionic acid from alanine, serine, lactate and other compounds; *Diplococcus glycinophilus*, which uses glycine as the sole source of energy (67); *Vibrio oxaliticus*, which is able to grow on oxalate, pyruvate, or acetate in an otherwise mineral medium (35); *Clostridium kluyveri*, which forms caproic acid from ethyl alcohol and acetate (20); and *Clostridium lactoacetophilum*, which produces butyric acid from lactate and acetate (34). Moore (274) has used the technique in the isolation of *Proactinomyces* which utilize pyridine. McClung (252) outlined techniques for isolation of chromogenic clostridia.

Single cell isolation.—In many research problems it is desirable

to establish without question the purity of the microbic strain under investigation by single cell isolation. In this connection it is of interest to note that the pneumatic micromanipulator of de Fonbrune is now available commercially in this country. Thaysen & Morris (371) described essential tools for micromanipulator use. Northcraft (281) and Soriano (341) have described simple methods for single cell isolation by the use of instruments which are available in the laboratory. Hildebrand (166) and Mason (265) have reviewed this problem.

Preservation of stock cultures and biological preparations.—In any laboratory dealing with a large number of species or varieties of living organisms there exists a major problem in keeping uniform stocks. With microorganisms it may be especially important to keep at a minimum changes in virulence, immunological properties, and morphological or biochemical characters (5, 48). For most microorganisms, the process of lyophilization is a satisfactory answer to this problem. Details of the process for molds were given by Raper & Alexander (303c) and for yeasts by Wickerham & Andreasen (401). Proom & Hemmons (300) outlined working details for a bacterial culture collection numbering 1,500 strains and suggested that the survival rate after exposure to 80°C. for one hour is a measure of the capacity of the particular batch of the dried culture to remain viable on storage at ordinary temperature. A variety of other suggestions have been made concerning the technique of lyophilization of bacteria (7, 121, 240, 268, 293, 305). Recent descriptions of apparatus for the desiccation of cultures include those of Gray (137), Heckly (156), and Rogers (323). Taylor & Beard (367) and Pomes & Irving (294) propose apparatus for large scale work. Warren (392) and Antoine & Hargett (6) described machines for shell freezing of small volumes of biological preparations such as yellow fever vaccine. Horsfall (181) designed a low temperature (−76°C.) storage cabinet for the preservation of viruses; Lofgren & Soule (239) found such a system satisfactory for *Spirocheta*.

Spore-forming bacteria and molds may be preserved on sterile soil using the general techniques outlined by Greene & Fred (139). Some bacteriologists (131, 152, 158, 276) prefer to layer cultures (nonspore-forming as well as spore-forming) with a small amount of sterile paraffin oil.

Microscopy.—Jelley (191) provided a review of recent develop-

ments in light microscopy. Of the instruments and methods discussed by him mention should be made of phase contrast microscopy which reveals details in transparent material having regions of slightly differing absorption or with different indices of refraction. Bennett *et al.* (32) discussed the theory of the method and the necessary apparatus, and Richards (311, 312) reviewed the applications in bacteriology and other areas in biology.

Hall (146) described a variable focus substage condenser which permits a finer focusing of the light source for the oil immersion and 4 mm. objectives as an aid in increasing resolving power and definition.

Cytological studies.—It is encouraging to note that the trend in studies of staining methods is to emphasize the mechanism of the staining reactions. This is particularly true for the Gram (15, 23, 159, 160) and acid fast (214, 422) techniques. It should be noted that the fluorescence technique of Hagemann (144, 145, 154) for the detection of acid fast organisms while preferred by some workers (182, 221, 231, 313, 314) is not acceptable to others (4, 119, 316). Indeed, Cummings (87) in a report on the laboratory diagnosis of the tubercle bacillus fails to include the technique.

An important advance in the understanding of the inner structure of bacterial cells, including the arrangement of chromatinic bodies, is reflected in the technique of Robinow (319, 320, 321) which employs extraction at 60°C. of cells, fixed with osmium tetraoxide or alcohol, with 1 *N* HCl and stained with Giemsa solution. Boivin's group (39, 375) have used ribonuclease and desoxyribonuclease treated cells with this technique to demonstrate the location of desoxyribonucleic acid in the central nucleus. Further information concerning the nucleus is given by Malmgren & Heden (258), who used ultraviolet absorption and the Feulgen technique, while Knaysi & Baker (209) secured evidence concerning the nuclei of *Bacillus mycoides* with the electron microscope. Subramaniam (357) gave details for handling yeast for cytological studies.

Pijper (290) has made a plea for more detailed studies of the morphology of bacteria or living cells, especially in the unstained state, and, with mounts in methylcellulose to slow down the motion, has made cinemicrographic records with sunlight darkground microscopy. His conclusions that bacteria move, not because of the action of flagella, but rather by a gyrating undulating move-

ment were not accepted by Conn & Elrod (78) nor Boltjes (42).

Electron microscope.—Within the past 10 years the electron microscope has become a valuable research instrument in many areas and especially in microbiology. General descriptions and bibliographies of the instrument and its uses are available (64, 82, 83, 123, 167, 207, 261, 263, 264, 420, 424), and in Burton's review (163) information may be found concerning recent advances in the construction of the instrument and its application. It is of interest to note that models operating at higher accelerating voltages are available (222, 262, 382) and judging by the micrographs of van Iterson (384) obtained with the Delft 100 KV model these instruments will indeed be of value. In addition to the standard preparations, the metal shadow casting technique (3, 407) has been applied to viruses (332, 409, 418) and bacteria (408). Although gold and other metals have been employed most commonly in this process, uranium or uranium oxide has been suggested (405). Several reports (12, 54, 168, 169) give details of preparation of microbiological specimens and the problem of conventional versus high speed microtomes for sectioning tissue material has been discussed (122, 286). In addition to the valuable data obtained on purified virus preparations, Nagler & Rake (279) suggested the use of the electron microscope in diagnosis of variola, vaccinia, and varicella by study of lesion material and Williams & Steere (406) have demonstrated tobacco mosaic virus in crude undiluted juice preparations. Wirth & Athanasiu (413) gave details of technique of culture on plastic membranes of mammalian cells for electron microscope study. Heinmets & Golub (157) presented material on the growth of the psittacosis virus in the chorioallantoic membrane, and Sharp (331) devised a method for counting virus particles in a suspension by means of the electron microscope.

Genetics of microorganisms.—Possibly no other field of experimental microbiology has been so active in late years as the study of spontaneous and induced mutations in viruses, bacteria and other fungi, and protozoa, as is revealed by various general reviews (24, 25, 26, 189, 217, 233, 234, 243, 340, 363) and by the proceedings of two symposia (132, 174, 232, 242, 342, 360). The importance of the studies on microorganisms to the general field of genetics can hardly be over-emphasized. Studies of the mutant strains reveal that many have altered morphological characters while others are unable to synthesize one or more essential metabolites or

have other alterations in their enzyme systems. With respect to technique of production of altered strains, various mutagenic agents have been used including x-rays and ultraviolet light for *Neurospora* (28, 178, 236, 327), *Penicillium* (44, 296, 304), *Aspergillus* (177), *Trichophyton* (108, 175, 176), *Ophiostoma* (120), *Absidia* (128), *Streptomyces* (203), *Rhodotorula* (46), *Azotobacter* (200), *Acetobacter* (136, 364), *Escherichia* (94, 136, 322, 364, 365), *Bacillus* (58), viruses (133); neutrons for *Penicillium* (149); acenaphthene for *Phytomonas* and *Erwinia* (8) and *Eberthella* (135); nitrogen mustard for *Neurospora* (45, 180, 254, 270, 362), *Escherichia* (56), and *Penicillium* (344) [see (257, 306) for methods for laboratory preparation and decontamination of mustard]; streptomycin for *Euglena* (301); antibodies for *Neurospora* (107); and lithium chloride for *Chromobacterium* (201).

Usually the exposure to mutagenic agents is done by exposure of vegetative cells, spores, or conidia in liquid suspensions but mutation is reported in *Aerobacter* exposed in the dry state (95). Mutants of *Escherichia* have been produced by treatment of the growth substrate prior to seeding (352, 353, 421). The great usefulness of the strains with altered biochemical reactions as research tools may be seen in the reviews listed above and, as an example of industrial importance of these mutants, it may be recalled that high yielding penicillin strains of *Penicillium notatum-chrysogenum* arose following exposure of the parent culture to mutagenic agents (303b). Lindegren (233, 234, 235) has reviewed the production of hybrid strains of yeast.

Spontaneous mutations have also been considered (163, 244, 245, 247, 423). McCarty (251) should be consulted for a review of the transforming principle of pneumococcal types.

Beadle & Tatum (27) described methods for the detection of mutants of *Neurospora;* Lederberg & Tatum (218) used plating in minimal agar of the inoculum containing possible mutants with addition, following incubation to allow unmutated cells to develop, of a complete medium to initiate growth of the mutants; Pontecorvo (295) used the auxanographic technique for detection of biochemical mutants and Fries (120) supplied a method for concentration of physiological mutants. Davis (89, 90) and Lederberg (219) have utilized the selective killing action of penicillin to isolate synthetically deficient mutants. Kelner (204) noted an effect

of visible light on the recovery of *Streptomyces* exposed to ultraviolet.

Metabolic studies.—Rapid strides have been and are being taken in the general field of cellular metabolism and much of the recent material relates to studies on microorganisms as shown in the reviews of Gunsalus (142) and Umbreit (377). For obtaining active enzyme preparations from bacterial cells several methods are available including the wet crushing mill of Booth & Green (47) and grinding with pyrex glass particles (196, 403), powdered alumina (255) or carborundum (308). Bellamy & Gunsalus (31) studied tyrosine decarboxylase from cells dried *in vacuo* over drierite. Lipmann (238) used preparations dried either with acetone or *in vacuo* over phosphorus pentoxide. Koepsell & Johnson (211) prepared extracts of cell pastes, obtained by Sharples centrifugation, of *Clostridium butylicum* by freezing and thawing, removing cellular debris by centrifugation, and lyophilizing the supernatant. Knight (210), in a study of the L-amino acid oxidase system of *Penicillium*, harvested the mycelium by filtration of liquid cultures through cotton and obtained active preparations by cold acetone extraction. These, when vacuum dried, retained activity for at least 10 days. Stumpf, Green & Smith (356) used ultrasonic disintegration of cells for preparation of extracts. In this connection, Shropshire (336) has described the instrument now available from the Raytheon Manufacturing Company of Waltham, Massachusetts. The techniques for Warburg manometric studies have been described by Umbriet *et al.* (378) and Dixon (98) and micromethods by Glick (130). Henry & Henry (161) give details for a turbidimetric method of following cell multiplication in Warburg flasks.

Stanier (345) appears to have made a fundamental contribution to the study of metabolic mechanisms in microorganisms by the use of simultaneous adaptation to possible intermediates in the reaction series. Woolley (416) reviewed the important topic of biological antagonism of structurally related compounds.

Radioactive tracer technique.—The use of radioactive tracers in experimental biology represents another important advance of the last decade. General problems and applications are discussed by Hevesy (164), Kamen (197), Sacks (325) and in the Wisconsin (361) and Cold Spring Harbor (359) symposia. Siri (338) compiled a handbook of methods (soon to be published in regular book form),

and others (66, 198, 411) give details of preparation and measurement of isotopic tracers. A review volume series has been initiated (216). Microbiological procedures for the preparation of several carbon-labeled fatty acids are supplied by Barker [see (66, pp. 271–77)]. Calvin *et al.* (66, pp. 346–48) give a bibliography up to July, 1948, of applications of tracer techniques to problems of microbiology. Some additional uses may be noted: use of isotopes in immunological investigations (49), studies on photosynthetic activity of purple bacteria and algae (2, 127), procedures for handling cultures containing radioactive materials (285). The significant work of Barker and his group illustrates the value of this technique in the study of the synthetic metabolic reactions of microbial forms. By use of the tracer technique, they have shown the synthesis of butyric and caproic acids from ethyl alcohol and acetic acid by *C. kluyveri* (18); carbon dioxide utilization in the synthesis of acetic and butyric acids by *Butyribacterum rettgeri* (19); carbon dioxide utilization in the synthesis of acetic acid by *Clostridium thermoaceticum* (17); and conversion of carbon dioxide to acetic and butyric acids and conversion of acetate to butyrate by *C. lactoacetophilum* (36). Another example of the use of isotopic tracer technique in bacteriology is found in the studies of nitrogen fixation (59, 60, 61, 412). Wood (414) has reviewed the use of isotopes in the study of carbon dioxide fixation, and Buchanan & Hastings (57) summarized the use of tagged carbon in studies of intermediary metabolism.

Microbiological assays.—The use of microorganisms as assay agents for determination of vitamins is now so common that it is necessary only to mention that the details of the techniques have been collected in book form (10, 192). The general technique can also be applied to the determination of amino acids (104). All of of these methods are based on the principle of the preparation of a basal medium adequate in all respects except for the test compound and the stimulation of the growth of the sensitive organism is correlated quantitatively with the addition of the test compound.

Antibiotics.—Waksman (389) has summarized the main techniques in current use for the enrichment of antibiotic producing organisms, and the procedures for assay of penicillin and streptomycin, at present the two most important antibiotics, have been summarized (297). Kelner (202) proposed a four-layered agar plate technique for detection of antibiotic producing species, and

Carlson & Douglas (68) suggested a screening method, involving solvent extractions, for discovery of antibiotic compounds in plants. To isolate streptomycin producing species, Waksman *et al.* (390) plated natural samples on a medium containing streptomycin and then tested the sensitivity of the actinomycetes which grew to an actinophage known to be active on streptomycin producing types. The use of test strains resistant to various antibiotics together with a calculation of their "inhibition ratios" was used by Stansly (347) as a means of identification of antibiotics in the crude state. In a search for possible antiviral agents, Jones & Schatz (193) used bacteriophages as the test agents.

Continuous culture methods and techniques for production of large quantities of cells.—Several reports have appeared concerning techniques and apparatus for continuous cultivation of microorganisms whereby, at intervals or continuously, sterile nutrients are supplied and samples or large volumes of the growth are drawn off. Related to this problem is the technique of submerged growth of aerobic organisms—a method which has been adapted successfully for industrial production of antibiotics and other fermentation products. Utilizing procedures which may be adapted to other organisms, an apparatus has been described for *Brucella* by Gerhardt (125) for continuous cultivation, and also details are available for submerged growth in a reciprocating shaker (126) or a sparger aeration system (124). Glassman & Elberg (129) also devised an aeration system for cultivation of massive amounts and indicate the general usefulness of the system in production of cultures of pathogenic bacteria. Kaplan & Elberg (199) devised a method for concentration of the cells of pathogenic types from broth cultures. Barnes & Dewey (21) grew *Shigella paradysenteriae* continuously at a rate to yield 4 gm. (dry weight) of cells per l. in two and one-half hours. Feustel & Humfeld (111) suggested a small laboratory fermenter employing mechanical agitation for dispersion of air introduced under pressure, and Humfeld (183) improved the agitation device to allow aeration at atmospheric pressure and added a mechanical foam breaker. Details are given for cultivation of yeast and *Bacillus subtilis*. Castor & Stier (74) suggested a simple flask system for continuous cultivation of yeast; Moor (272) utilized a horizontal tube, for growth of *Penicillium* and yeast, which can be aerated and from which samples can be obtained at intervals, and Lewis & Lucas (230) described a

cellophane tube system for flowing medium studies. Further details are available for laboratory production of penicillin (117, 277) and amylase (16, 385) by submerged cultures. For molds the use of fragmented mycelia as inocula has been suggested to conserve materials and hasten culture production (100, 328). Owen (283) devised a multiple-deck system for continuous cultivation of yeast. Harmsen & Kolff (150) utilized cellophane membranes as an aid in production of large quantities of cells, and Brown & Wood (55) grew *Brucella* in broth in centrifuge bottles containing blocks of cellulose sponge and collected cells by centrifugation. Stansly *et al.* (348) reported details of a system, which could be used for other purposes, for large scale laboratory production of polymyxin; Olson & Johnson (282) described apparatus for acid hydrolysis of wheat mash for media for a slow feed culture system. Shwartzman & Bierman (337) discussed sampling of cultures and also gave a technique for even distribution of gases through bacterial cultures. Hart & Blumberg (151) also reported details for obtaining samples of culture without disturbing growth conditions, and Hunt (184), in a study of growth of excised plant roots, used an aeration system which did not allow contamination. Problems of aeration of liquid cultures relating to size of orifice of sparger have been presented (1). Jordan & Jacobs (194) studied the growth of *E. coli* with a constant food supply. Bilford *et al.* (37) described the industrial process for producing ethyl alcohol by continuous yeast fermentation.

Virus techniques.—It is considered that the techniques pertaining to the study of viruses are beyond the scope of this summary. It may be mentioned in passing, however, that many of the procedures are discussed in the critique of Beard (29) on purified animal viruses, other general reviews (172, 291, 318, 326, 346, 419), books (223, 224, 225, 317, 386), or the summaries relating to the bacterial viruses (85, 92, 93, 116). The new report of Beveridge & Burnet (33) gives details of the embryonated egg technique for cultivation of viruses and rickettsia.

Chromatography.—A technique of considerable interest to microbiologists concerned with the problem of separation of closely related chemical substances is that of paper chromatography introduced by Consden, Gordon & Martin (80) for the separation of amino acids. The method has been used for a wide variety of substances including chicken tumor virus (315), vitamins (113), penicillins (30, 113, 208, 373), streptomycin (179, 287), sugars

(284), flavin nucleotides (86), and histamine (380). X-ray and electron diffraction methods of analysis may aid in identification of small quantities of material separated by chromatography (75, 298). The method is also useful in radioactive tracer studies (112, 206). Those interested in the method should consult the review of Strain (354), the symposium edited by Cassidy (73), and the recent paper of Consden (79). Martin (260) gives a general introduction. Partition chromatography also has been used for the estimation of fatty acids in biological materials (106, 278, 288, 303a).

Chemical and physical methods and apparatus.—Many of the presently important problems in microbiology require the use of methods or apparatus formerly thought to be in the domain of physics and chemistry (81, 395). The use of the electron microscope discussed above, is an example. With reference to other techniques it is of interest to note that the Federation of American Societies for Experimental Biology held a symposium, in March of 1948, titled "Methods for Determination of Purity of Substances of Biochemical Interest." This included discussions of chromatography (72), counter-current distribution (84), diffusion, sedimentation, and electrophoresis (404), and solubility methods of analysis (162). Lauffer [(215), see also (317)] discussed some of these techniques and ultrafiltration in relation to studies of viruses. Shedlovsky (333) considered the criteria of purity of proteins.

Moore & White (273) published details of the new compact Tiselius apparatus now manufactured in this country by the Perkin-Elmer Corporation; Stern has designed recently an additional model which is manufactured by the American Instrument Company. Additional information concerning the Tiselius apparatus is available (11, 213, 227, 249, 374). The ultracentrifuge is another instrument of value in the study of proteins (96, 97, 248). Photoelectric measurement of densities of suspensions of microorganisms or other biological preparations is a frequently used technique. Several recent papers include descriptions of new instruments or are concerned particularly with the use of them (14, 41, 43, 51, 212, 280, 379, 383). Griner, Tytell & Kersten (140) gave details concerning a luminometer for measuring bacterial luminescence. Lewis & Hayden (229) described a mass spectrograph for separation of small quantities of radioactive isotopes. Heatley (155) designed a new type of microrespirometer requiring

about 1/200th of the amount of experimental material needed for the conventional Barcroft or Warburg apparatus. New ultrasonic disintegrators have been described (336, 339, 356).

Miscellaneous laboratory techniques and minor apparatus.—The problem of cultivation of anaerobic bacteria in media exposed to atmospheric oxygen was solved by Brewer (53), who proposed sodium thioglycollate as a nontoxic reducing agent. Utilizing this compound, media have been prepared which serve satisfactorily in the diagnostic clinical laboratory (115, 307) and in the general taxonomic study of this group (387). Likewise such media are proposed for general sterility testing (292) and specifically for testing of sterility of catgut (76) because of the neutralization by the thioglycollate of mercurial compounds. Ulrich & Larsen (376) suggest a single solution to serve as an indicator of anaerobiosis. Hewitt (165) released a new edition of the monograph on oxidation-reduction potentials.

Weaver and his students have proposed a series of quick micro-technique reactions for qualitative determination of production of indole (9), acetyl methyl carbinol (109), hydrogen sulfide (275), and reduction of nitrates (13) and carbohydrate fermentation (148). A variety of other techniques reported recently include methods for: obtaining standard single cell suspension of tubercle bacilli by ball mill grinding (396), diagnosis of tuberculosis by mouse inoculation (271), use of phosphatides of long chain fatty esters of polyhydric alcohols to encourage rapid submerged growth of mycobacteria (101, 102), use of such media for isolation of *Mycobacterium tuberculosis* from pathological samples (114), manometric determination of galactose in presence of other sugars (343), studying thermal resistance of spore-forming bacteria (355), isolation of Listerellae from bovine brain material (138), assay of antipernicious anemia factor with *Euglena* (186), evaluation of quaternary ammonium compounds as sanitizing agents for food utensils (393). Special tubes have been designed to determine carbon dioxide/hydrogen ratios (250) and to permit isolation of motile monophasic from diphasic *Salmonella* (226).

Grossowicz & Kaplan (141) indicated that certain bacteriological media may be sterilized chemically by addition of mercuric oxycyanide and subsequent neutralization of this compound by thioglycollate. Dunn *et al.* (103) sterilized fluids by a continuous process using high energy roentgen and cathode rays. Carlson,

Hollaender & Gaulden (69) used ultraviolet light as a means of sterilizing tissue culture materials. Hutner & Bjerknes (185) suggested for biological solutions effective preservatives which volatilize on steam sterilization. Sterges (349, 350) and others (187, 188, 368) gave details for preparation of silica gel for bacteriological studies. Surveys on the proper medium for cultivation of *Neisseria* have been made (70, 370).

Phaff & Mrak (289) reviewed the literature on methods for yeast sporulation. An evaluation has been made by Wickerham (400, 402) of the carbon and nitrogen assimilation tests for classification of yeasts, and Pridham & Gottleib (299) studied utilization of carbon compounds by Actinomycetales. Production of lecithinase has been used in classification of species of *Clostridium* (253) and *Bacillus* (77). The use of bacteriophage for classification of bacteria has been reviewed (85, 110).

Hirsch (171) suggested an apparatus for roll-tubes to permit substitution of them for petri plates in counting viable bacteria. Hoffstadt & Tripi (173) designed small stainless steel jars which may be used with the conventional Waring blendor for maceration of microsamples.

Wood (415) proposed a dark field colony illuminator. Valuable information concerning photomicrography may be found in articles by Shillaber (335) and Boltjes (40).

Ultraviolet irradiation has been suggested as the inactivation agent for the production of immunizing antigens from toxins, viruses, and bacterial cells (52, 143, 228). Inactivation of viruses by exposure to mustard has been proposed (369).

White (398, 399) has reviewed methods for cultivation of animal and plant tissues.

Air-borne infections and devices for protection of the investigator.—Intensive study, stimulated by the recent world conflict, of induced and natural air-borne infections, has opened a variety of problems such as the design and operation of spray chambers and the sampling of air. These are discussed by Rosebury (324) in a monograph which summarizes a war research project. Two additional reports (334, 381) are concerned with the details of construction of inoculation cabinets designed to minimize infection as a result of handling pathogenic species. A notable advance in laboratory construction, such as the Infectious Diseases Building of the National Institute of Health, takes into account the need for

protection of personnel in specialized research areas. Certain aspects of such laboratory designs are presented by Bogue (38). Lurie & Abramson (246) tested the efficiency of gauze masks on rabbits exposed to air-borne tuberculosis organisms. Luckiesh *et al.* (241) discussed sampling devices for air-borne organisms.

Rearing of laboratory animals.—In many projects the supply of laboratory animals is a critical factor and all who have this problem will find useful the general book compiled by Worden (417) which summarizes available information concerning laboratory breeding of the common, and many of the uncommon, animals which might be used in research. Additional reports are concerned with hamsters (205), cotton rats (269), wild mice (330), fleas (62, 220), mosquitoes (88, 220), and other insects (394).

Quite apart from the problem of the supply of animals in the usual sense (and yet closely related to it) is the rearing of laboratory animals under germ-free conditions. Reyniers (310) summarized the experiences of the Notre Dame Laboratories and further material will be found in *Lobund Reports* (309), an occasional publication from the same source.

Statistics.—There are so many areas in microbiology in which accurate statistical analysis of research data is needed that listing of even representative problems seems superfluous. Fortunately, recent reviews (105, 372, 397) are available to serve as a guide to this general problem and publication is expected of the January, 1949, symposium of the New York Academy of Sciences (91). Halvorson & Ziegler (147) give useful information on quantitative bacteriology.

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BACTERIOPHAGE[1,2]

By J. Fong
Department of Bacteriology, University of California, Berkeley 4, California

Ever since the discovery of the phenomenon of bacteriophagy by Twort (1) and by d'Herelle (2), speculation has arisen regarding the nature of bacteriophage, its mode of action, and the mechanism of its reproduction. Today there is considerable evidence that the bacteriophages possess most of the properties commonly ascribed to the animal and plant viruses. The recent investigations based upon biochemical, physiological, biophysical, and genetical principles have found answers to some fundamental questions and have opened up promising approaches to still others. The present review makes no attempt to survey the entire field of phage research but is limited in scope to certain aspects of the subject which appear to the writer to possess particular significance.

Morphology

Clearly defined observations on the morphology of the bacteriophages only recently have been reported, for adequate demonstration of the bacterial viruses depends upon satisfactory methods of observation, a possibility now realized with the electron microscope. Ruska (3, 4) described the presence of club-shaped structures resulting from the action of phage upon nonspore-forming bacteria and presented very distinct micrographs of particles of varying size and shape lying in close proximity to the cells from which they were presumably derived. Similar descriptions of these so-called d'Herelle bodies were reported by Kottman (5).

The morphology of certain bacteriophages active against *Escherichia coli* has been fully described by various workers (6 to 9); they have been shown to be either sperm-shaped bodies or spherical structures without tails. The phages which have been investigated most thoroughly are the seven phages designated as T1, T2 . . . and T7. The morphologically identical viruses, T2, T4, and T6, all possess head and tail structures. On the other hand, the T3 and

[1] This review covers the period from January, 1941 to February, 1949.

[2] The writer wishes to thank the members of the Department, Drs. A. P. Krueger, R. Y. Stanier, M. Doudoroff, and S. S. Elberg, for their many valuable suggestions and criticisms. He is also indebted to Drs. W. M. Stanley and Dean Fraser for reading the manuscript and for offering many helpful comments.

T7 viruses simply consist of approximately spherical bodies without tails. While the T1 and T5 viruses possess both head and tail structures, certain morphological differences such as size and density of internal structure are apparent between these two phages.

Studies on the dimensions of these viruses have demonstrated that the T2 phage obtained from lysates in broth exhibits a total length of 211 mμ and a head 80 mμ wide; the corresponding dimensions for the phage concentrated from lysates in synthetic media are 248 mμ and 86 mμ respectively (10). The T3 and T7 viruses are approximately 45 mμ in diameter. The particles of the T1 phage consist of round dense heads, 45 to 50 mμ in diameter, and extremely thin tails approximately 150 mμ long and not more than 10 to 15 mμ thick. The T5 virus possesses a head 90 mμ wide and a tail 170 mμ long by 15 mμ wide (8, 9).

Further investigations (10), particularly with the T2 bacteriophage, have demonstrated that the constituents of the virus particles appear to be arranged in a highly organized and differentiated manner. The material within the headpiece exhibits not only segregated regions of high and low electron-absorbing power but also differentiation from a limiting membrane. The loss of the internal constituent leads to forms consisting only of the external membrane and attached tail, the so-called ghosts of the virus particles (9, 10).

Sharp *et al.* (11) on shadowing the T2 virus preparations with chromium observed that the heads do not give shadows characteristic either of ideal spheres or even regular polyhedrons, but rather those of short, somewhat flattened rods with conical caps. Unfortunately, it was not possible to estimate the degree of shrinkage and distortion on drying, a consideration of obvious importance (12). The stubby tail, of uneven thickness and terminating in a ball- or disc-shaped structure, likewise cast short shadows indicating a flattened condition (11).

Electron micrography has shown rather similar structures for other bacteriophages, and either round particles or tadpole-shaped structures with varying degrees of internal differentiation have been described for coli (7), staphylococcus (6, 7, 13), and pyocyaneus (14) phages. The pyocyaneus phage 238 consists of a pentagonal head with an average diameter of 76 to 85 mμ; the head contains a dense, filamentous internal structure which apparently forms a continuous loop. The straight tail is 150 by 15 mμ (14).

It is of significant interest to find that phages active upon actinomycetes have been reported recently (15, 16), and that they approximate some of the better known phages in both size and shape. However, they differ from the latter in that many of the actinophage particles described by Woodruff *et al.* (16) are composed of two distinct bodies; a few appear to be similar to tetrads, and one or two preparations even show a majority of particles with two tails.

Chemical Composition

Recently several reports have been published describing the chemical composition of the T2 bacteriophage (10, 17, 18, 19). In a preliminary study, Hook *et al.* (10) reported that the T2 bacteriophage contains both ribo- and desoxyribonucleic acids. The observations have been extended by Taylor (18) in a detailed analysis of the elementary and molecular composition of T2 bacteriophages obtained from lysates of cultures in broth and in synthetic media. Prior to analysis the phages were concentrated and purified by differential centrifugation. His data revealed the presence of protein, nucleic acids, and a small amount of lipids. The protein content, expressed in percentage of dry weight, amounted to 50.6 per cent for lysates from broth and 52.4 per cent for lysates from synthetic media. Analyses of the nucleic acids showed that both the ribose and desoxyribose types were present, the latter predominating. The total content of nucleic acid from lysates in broth and in synthetic media was not significantly different, the values being 46.9 and 45.9 per cent respectively; however, the distribution of the two varieties of nucleic acid was markedly dissimilar, the content of ribonucleic acid being five times greater in the preparations derived from lysates in broth.

Cohen & Anderson (17) also investigated the chemistry of T2 phages produced in cells grown in synthetic media. They were able to detect only the desoxyribose type of nucleic acid, their value of 36.9 per cent being somewhat lower than that reported by Taylor. Cohen (19, 20) has raised some cogent objections to Taylor's findings and has concluded that ribonucleic acid is an unlikely constituent of the T2 particles, since at least 99 per cent of the total phosphorus in his own preparations could be accounted for by the desoxyribonucleic acid.

Taylor (18) found a lipid content of 2.6 and 1.77 per cent respectively for the lysates from broth and from synthetic media.

The small amount of lipid present did not contain nitrogen or phosphorus, indicating the absence of phospholipids; since cholesterol was likewise absent, Taylor regarded the fraction as neutral fat.

It is of some interest to find that the medium upon which the host is grown may influence not only the gross chemical composition of the virus but also the location of the chemical component in the virus. Thus, T2 virus grown on *E. coli* in a synthetic lactate medium and subsequently concentrated by the usual procedures appears to possess a coating of sedimentable desoxyribonucleic acid capable of reacting with streptomycin and resulting in lattice formation and precipitation. The same virus propagated in cultures in nutrient broth does not show an external coating of desoxyribonucleic acid, because the latter is broken down by the desoxyribonuclease which is present in lysates obtained from broth cultures (21).

The chemical composition of a staphylococcal phage has been reported by Northrop (22). Following the chemical procedures previously employed for the purification of enzymes, he isolated from staphylococcal lysates a macromolecular nucleoprotein having the activity of bacteriophage and possessing a chemical composition quite similar to those reported for the coli phages. Analysis of his phage preparations disclosed 14.1, 5.0, and 1.5 per cent of nitrogen, phosphorus, and carbohydrate respectively.

The bacteriophages, therefore, are apparently complexes consisting of protein, nucleic acid, and lipid. In constitutional complexity and fundamental chemical make-up they resemble their bacterial hosts, though certain qualitative and quantitative differences are observable, especially in the kind and amount of lipid present and in the distribution of the two kinds of nucleic acid (18). If Cohen & Anderson's observations (17) are correct, and there is only one type of nucleic acid present, then the chemical composition of the bacteriophages places them more closely in line with the animal viruses, for most of the latter viruses thus far studied generally contain only the desoxyribose type of nucleic acid. The bacteriophages, however, differ from the animal viruses in that they do not appear to possess phospholipid or cholesterol, the lipid being present solely as neutral fat (18). These conclusions eventually may have to be revised, for Pirie (23) has concluded that few virus preparations have been examined

critically for contamination and that techniques usually adopted for demonstrating homogeneity are not well adapted for the recognition of low levels of contamination.

Mechanism of Virus Growth

Studies on the growth of bacteriophage have demonstrated the existence of three well-defined phases, namely, adsorption, latent period of intracellular reproduction, and finally, release of newly-formed phage (24).

Adsorption.—The rate of adsorption of virus upon susceptible host cells depends on a number of factors such as concentrations of virus and cell, temperature, and concentration of electrolytes (25, 26, 27). Electrolytes in proper proportions expedite adsorption but excessive amounts may hinder this process, the inhibitory effect being greater at higher temperatures (27). The effect presumably is a result of an altered cell surface, for high concentrations of sodium chloride concomitantly protect the phage-augmenting capacity of rapidly-grown staphylococci so that not only is contact between phage and susceptible cells prevented, but apparently the heat lability of such cells is also decreased (28). The strain of susceptible bacteria employed (29) and the physiological state of the host cells (30, 31) likewise exert an influence on the ability of sensitive cells to absorb virus.

Anderson (32) has shown recently that the T4 and T6 bacteriophages require the presence of certain aromatic amino acids, particularly L-tryptophane, for efficient adsorption. The activation of virus by L-tryptophane is a function of the concentration of cofactor, time of exposure, hydrogen ion concentration, and temperature (33). A small fraction of the T4 particles propagated on host cells incubated at 37°C. does not require preliminary activation, because apparently enough cofactor is produced by the bacterial host to activate the virus (34). The bacteriophages T1 and T7 similarly require certain amino acids such as isoleucine, norleucine, and methionine as adsorption cofactors (35).

Delbrück (36) has suggested that adsorption may actually occur in two steps, involving first a physical adsorption that is static in nature, and secondly the "invasion" of the host cells, an enzymatically controlled process. There are certainly several features indicative of an enzyme-like activity as, for example, the increased rate of adsorption at higher temperatures (26), the speci-

ficity of L-tryptophane in accomplishing the activation of the T4 and T6 viruses (33), and the fact that the activity of L-tryptophane may be blocked in certain instances by small amounts of an antimetabolite such as indole (36).

Latent period of intracellular virus growth.—In the *E. coli*-phage system when the bacterial host has adsorbed virus, certain reactions are set in motion, resulting in experimentally determinable changes within the virus-infected host cells. Thus, infected cells no longer undergo cellular division even though respiration continues, and the normal metabolic activities of the infected cells are considerably altered so that there is now an increased rate of desoxyribonucleic acid synthesis.

The failure of infected cells to divide (17, 37) does not appear to interfere with the process of virus multiplication, for Spizizen (38) has observed phage reproduction in cells of *E. coli* inhibited by glycine anhydride, and Anderson (39, 40), as well as other investigators (41, 42), has shown that bacteria irradiated with ultraviolet light while no longer capable of multiplication are, nevertheless, able to support virus growth. More recent evidence by Price (43, 44, 45), Krueger *et al.* (46, 47), and Elford (48) has shown that concentrations of penicillin sufficient to suppress bacterial growth will not prevent phage formation. Under these circumstances, the extent to which phage is produced is influenced very little by the original penicillin concentration but is markedly dependent upon the initial phage concentration, for smaller initial concentrations of phage lead to greater yields on a percentage basis (47). In a similar manner, bacteria rendered nonviable by mustard gas are still capable of supporting virus growth (49).

Monod & Wollman (50) have reported that a phage-infected culture, when suspended with an organic substrate attacked by a constitutive enzyme, will exhibit virus reproduction. However, when the same suspension is placed in the presence of a substrate which is attacked only after the formation of adaptive enzymes, no virus reproduction ensues. These findings indicate that phage infection of susceptible host cells results in inhibition of adaptive enzyme synthesis and that this in turn restricts phage multiplication.

It is evident, then, that the complicated series of enzymatic reactions essential for cell multiplication are dispensable for virus synthesis, but it is equally obvious that certain other metabolic

reactions are required since infected cells continue to show a constant rate of oxygen consumption as well as a respiratory quotient not significantly different from that of cells just before infection (17). Further evidence for this belief is the fact that various metabolic poisons will inhibit phage reproduction (45, 51) whereas, coenzymes and metabolites known to be intermediates in cell metabolism, such as yeast nucleic acid, will enhance virus multiplication (51, 52). This stimulatory substance is taken up by the bacteria, and in the absence of virus, the material is metabolized to a form no longer available for virus formation. A similar stimulant of virus reproduction can be isolated from normal *Staphylococcus muscae* cells, and it seems probable that a certain concentration of this compound is normally present in the host cells (52). Even an inorganic ion such as calcium may be essential for the growth of some types of phages (53).

The effect of essential metabolites is still more strikingly demonstrated by the series of investigations carried out by Cohen & Fowler (54, 55, 56). Apparently tryptophane is such a metabolite since it specifically reverses the inhibition of virus multiplication by the antimetabolite 5-methyl tryptophane (54). In addition to tryptophane it appears that twelve other amino acids are necessary (56), and the rate and amount of virus synthesis normally occurring in nutrient broth can only be approximated in a deficient medium by the addition of a complex mixture of L-amino acids and purine and pyrimidine bases (55).

During the course of normal growth the cells of *E. coli* synthesize almost three times as much ribonucleic acid as desoxyribonucleic acid (18, 19); however, susceptible cells suspended in a synthetic medium and infected with T2 virus will show a rate of desoxyribonucleic acid synthesis approximately four times as great as that observed in normal cultures (19, 57). The increment in phosphorus content of infected cells can be quantitatively accounted for by the increment in desoxyribonucleic acid, indicating that the latter is the only protein-bound, phosphorus-containing constituent produced by infected cells. Any procedure that causes inhibition of virus multiplication as, for example, treatment with 5-methyl tryptophane or infection with ultraviolet-irradiated virus results also in suppression of desoxyribonucleic acid synthesis. Of significant interest is the fact that synthesis of protein commences from the moment of infection, but the synthesis of

desoxyribonucleic acid does not begin for about 8 to 10 min. (19, 57). This lag in the production of desoxyribonucleic acid or virus agrees very closely with the period of approximately 7 min. reported by Latarjet (58) duting which no virus reproduction takes place intracellularly.

Cohen (20) has investigated the origin of the virus phosphorus and has concluded that the ribopentose type of nucleic acid, as indicated by tracer studies with radioactive phosphorus, was not the precursor material for desoxyribonucleic acid. His findings indicated that the virus phosphorus was derived wholly from the environment. However, subsequent studies by Putnam & Kozloff (59) suggested that the origin of an appreciable fraction of the virus phosphorus may arise from sources other than the medium.

Another approach to a study of the intracellular growth of virus is based on the fact that adequate doses of ultraviolet- and x-irradiations can cause inactivation of both extracellular and intracellular phage. Thus, Luria & Latarjet (41), using ultraviolet irradiation, have shown that in the first 12 min. following infection the infected bacteria show a rapid increase in resistance to irradiation; at later times, however, the resistance of infected bacteria to high doses of radiation decreases. Subsequently, Latarjet (58) demonstrated the same series of phenomena with x-irradiations. On the basis of his experiments he divided the latent period of intracellular virus growth into three parts. For the first 7 min. the resistance to radiation of most of the infective particles remained constant. Between 7 and 13 min. resistance to radiation increased and the survival curves of infective centers progressed to the multiple-hit type. Finally, from 13 min. to the moment of burst there was an increasing radiation sensitivity.

These findings with ultraviolet- and x-irradiations, when taken in conjunction with the preceding biochemical data, afford some insight into the mechanism of intracellular growth of virus. It appears that for the first 7 or 8 min. there is no virus multiplication though presumably virus peptides are being synthesized through the metabolic activities of the host. At the end of this period virus reproduction begins and continues to the thirteenth minute at which time it stops, possibly because of the exhaustion of some limiting substrate. The period from 13 min. to the time of burst may represent the time necessary for maturation of the newly-formed virus particles. Electron micrographic studies by Wykoff

(60, 61, 62) lend credence to this concept, since they show that the amount of material within a phage head increases as the particle matures. Thus, recently infected cells simply exhibit holes in the cytoplasm, their connection with phage being demonstrated by the tails present. In contrast, longer-diseased cells show more substance within these holes.

Interference Phenomenon

The interference phenomenon may express itself when a bacterium is simultaneously infected with two or more bacteriophages; when this occurs, only one of the phages will grow, and the bacterium will be lysed after a time interval characteristic for the virus which grows. The growth of only one type of virus has been designated as the mutual exclusion effect. It appears that the virus which fails to grow may exert a so-called depressor effect so that the yield of the virus which grows is reduced.

The mutual exclusion effect was first demonstrated by the study of mixed infections (63). Thus, when *E. coli* is infected with T2 virus any time up to 4 min. following exposure of the bacterium to T1 virus, the T2 virus will suppress completely the growth of T1 virus. In the case of T1 and T7 viruses, Delbrück (64) found that for equal multiplicity of infection with both viruses, about one-third of the bacteria liberates T1 virus while two-thirds liberate T7 virus. The same phenomenon may be demonstrated using ultraviolet-inactivated particles of T2 as the excluding agent; in this respect Luria & Delbrück (37) have shown that properly irradiated T2 virus will suppress the growth of T1 virus.

Cohen & Anderson (65) have found that a chemical substance, 5-methyl tryptophane, is capable of altering the bacterial cells in such a manner as to render them incapable of supporting virus growth; however, this chemical interference differs from the other two types of interference in that the effects of the chemical could be reversed if its action is not too greatly prolonged.

The mutual exclusion and depressor effects are not always absolute, for Hershey (66) has found that the majority of bacteria mixedly infected with wild-type phage and its *r* mutant yielded plaques containing both phages without any demonstrable reduction in virus production. Subsequent investigations, however, indicate that the mutual exclusion effect is complete with unrelated viruses but that with pairs of related viruses such as T2, T4, and

T6 bacteriophages, there is partial but not complete mutual exclusion (67).

While various hypotheses have been advanced (63, 64) there does not appear to be any completely satisfactory explanation for the interference phenomenon at present. The data obtained from ultraviolet and x-ray irradiations indicate that several virus particles are capable of growing in the same bacterial cell, a fact in accordance with Hershey's observation (66) that bacteria simultaneously infected with T2 wild-type and its *r* mutant give rise to both types. However, since infection leads first to alterations in the metabolism of the host, it seems likely that the interference phenomenon results from changes in host functions.

Host Mutations from Virus Sensitivity to Virus Resistance

The origin of bacterial variants that prove resistant to the action of a bacteriophage capable of lysing the original parent culture has been the subject of considerable debate. Both Gratia (68) and Burnet (69) considered that the phage-resistant bacterial variants stem from mutations which occur independently of the bacteriophage, and that the latter merely brings the resistant cells into prominence by eliminating all susceptible bacteria. Burnet has succeeded in actually isolating colonies which were morphologically different and which subsequently proved resistant to the action of phage.

Evidence of a more convincing nature has been advanced by Luria & Delbrück (70). If phage-resistant mutants arise only after contact with the homologous phage, then the resistant colonies which develop in a series of similar cultures after exposure to phage should be constant in numbers, since all susceptible cells are subjected to an equal chance of destruction. On the other hand, if these mutants arise spontaneously, they should exhibit considerable fluctuations in numbers, depending on the time at which a mutation first occurs. The latter thesis was experimentally demonstrated for *E. coli*, strain B, and the T1 bacteriophage (70).

The T set of bacteriophages has been divided into four cross-resistance groups consisting of T1 and T5; T3, T4, and T7; T2; and T6. Cells that prove resistant to any one phage may also be resistant to other phages within the same cross-resistance group. Thus, mutant cells isolated in the presence of T7 phage, for example, are

also insensitive to the T3 and T4 phages as a rule. Rarely are mutants encountered which prove resistant to phages in different resistance groups (71).

Phage-resistant mutants are designated according to the nomenclature proposed by Demerec & Fano (71). For example, if a resistant mutant is obtained as a secondary growth after plating susceptible cells in the presence of T1 phage, it is designated as B/1. A mutant labelled B/3, 2, 4, 7 indicates a bacterium selected for resistance to T3 but proving resistant also to T2, T4, and T7.

Calculations of the rates of mutation from phage sensitivity to phage resistance by the formula of Luria & Delbrück (70) have given somewhat variable results for any one mutation. One possible explanation for these discrepancies has been given by Beale (72), who concluded that the mutation rate is a constant function of cell division, and that a mutation rate of 0.7 per 10^8 bacterial divisions for the mutation to resistance against T1 phage can be obtained only under constant conditions, namely, by using one particular culture of bacteria and one particular culture of phage, and confining the observations to the period during which the bacteria are in an actively dividing state. Newcombe (73, 74), on the other hand, has ascribed the variations in values to a delay between a genetic change and its phenotypic expression. By comparing the rate of appearance of phenotypically resistant clones during bacterial multiplication with the rate of gene mutation, the latter being estimated from the numbers of resistant individuals arising during similar periods of growth, Newcombe has found that the rate of appearance of resistant clones per bacterium per division cycle is approximately 0.5×10^{-8} whereas the rate of gene mutation is 3×10^{-8}.

In addition to the spontaneous mutations, it has been demonstrated that the rate of mutation from phage sensitivity to phage resistance in any normal, susceptible bacterial culture may be increased by ultraviolet- and x-irradiations (75, 76), or by the action of chemical substances such as sodium desoxycholate, pyronin, acriflavine (77), and nitrogen mustard (78).

Mutations to phage resistance are sometimes accompanied by metabolic changes; Luria (79) has found that certain mutations of the host cells from virus sensitivity to virus resistance result in a slower growth rate for the mutant cells. Anderson (80, 81) has shown that some B/1 mutants of *E. coli* have lost the ability to

synthesize L-tryptophane while a strain of B/1, 3, 4, 7 has become incapable of synthesizing the amino acid proline. Wollman (82) has reported similar results with *E. coli*, strain CB, and various other workers (83, 84) have described analogous metabolic changes correlated with mutations of this type.

Recently two specific mechanisms underlying phage resistance have been demonstrated. Anderson (35) reported the existence of two types of T4 virus, one of which required adsorption cofactor, while the other did not, presumably because the latter was able to utilize the small amounts of cofactor produced by the metabolizing host cells when grown at the usual optimal temperature. At lower temperatures, however, there was insufficient production of cofactor by the host cells, resulting in inadequate activation of virus, and, thus, in turn leading to the absence of adsorption and eventual lysis. In this instance, then, the physiological basis for sensitivity at the higher temperature of 37°C. resulted from the fact that the host cells synthesized and liberated enough cofactor to activate the virus; on the other hand, resistance at the lower temperature of 14°C. was related to the fact that less cofactor was liberated. The case studied by Delbrück (36) showed that certain strains of T4 virus required adsorption cofactors but that the activation by the cofactors could be effectively blocked by indole; consequently, when the bacterial hosts liberated indole during their metabolism of tryptophane, resistance against T4 phage would result.

Virus Mutations

Several different types of viral mutants have been described; this work has been admirably reviewed by Hershey & Bronfenbrenner (85). The first quantitative examination of a specific viral mutation was carried out by Luria (86, 87) though various types of phage variation had been described previously (88 to 91). By plating T2 phage with a resistant bacterial strain B/2, Luria obtained a new virus T2*h*, differing from the parent T2 phage by its ability to attack the cells of B/2. In a similar manner the virus strain T1*h* was established (86). The mutational origin of these two new viruses was demonstrated by analyzing the distribution of the number of T2*h* or T1*h* plaques produced on resistant bacterial strains. Several varieties of these virus mutants exhibiting an altered host range have been described (66, 92). Thus, the wild-

type phage T2H may give rise to T2Hh^t (h^t for turbid plaques), T2Hh^c (h^c for clear plaques), and T2Hh^b (h^b differs from h^c in being able to lyse B/2, 2H, h^c, which is resistant to h^c virus).

Viral mutants relating to type of plaque have been described for the morphologically and serologically related viruses T2, T4, and T6 (66, 92). All these phages exhibit the phenomenon of lysis inhibition as manifested by the fact that on addition of phage to a heavy, turbid bacterial culture in amounts sufficient to infect all or most of the cells lysis occurred only after 5 to 6 hr., although a sample of the same bacterial culture previously diluted lysed promptly 21 min. after the addition of phage. Doermann (93) has shown that the phenomenon of lysis inhibition can only be elicited if bacteria infected by wild-type (r^+) particles are reinfected for the second time by one or more r^+ particles of the same or similar type.

Lysis-inhibiting virus strains are able to give rise to the r mutants exhibiting no lysis inhibition (66). These r mutants, which usually appear as clear sectors in the partially lysed halos surrounding plaques of the wild-type, produce on isolation a distinctive type of plaque characterized by nearly complete lysis at the periphery. The r mutations occur in an identical manner in all the even-numbered phages thus far studied, the rate of the mutation being approximately one per thousand duplications of virus. Back mutations from r to wild-type may also occur (frequency of one in every 10^8 bacterial divisions), and because the r mutants show a definite competitive disadvantage when grown together with wild-type, they are generally replaced by the latter despite the unfavorable mutation rate obtaining with wild-type. The mutation from r^+ to r occurs without any alteration in host or antigenic specificity.

Mutations affecting host range and lysis inhibition are determined by separate genetic sites (92), the expression of either one not being influenced by the occurrence of the other. This is evidenced by the identity of mutation rates for the transformation from h^+ to h^c in both r and r^+ stocks of T2H and by the fact that analyses of their mutational patterns reveal that mutations to r and reversion to r^+ can occur irrespective of the existing h phenotype (for example, T2Hh^c may mutate to T2Hh^cr or the latter may revert to the former without any change occurring in the h loci).

Additionally, each of these characters (host range and lysis in-

hibition) seems to be determined by several independent loci, each locus consisting of a single structure subject to reversible alternation between only two alleles (92). Substantiation for this is found in various observations. Thus, the host range mutations from h^+ to h^b and from h^c to h^ch^b (obtained by selecting h^c virus active on B/2, 2H, h^c) occur with a similar frequency. Furthermore it is possible to separate these genetic factors, for bacteria mixedly infected with h^+r and $h^ch^br^+$ were found experimentally to give rise to all but one of the eight possible phenotypes theoretically obtainable if h^b and h^c represented independent genetic factors; the single exception is the mutant h^br^+, and the failure to demonstrate this phenotype may be related to its poor growth on sensitive bacteria. Additional evidence for the existence of several loci governing host range mutations is afforded by the study of back mutations; in this respect, it can be demonstrated that the mutant h^b readily reverts to h^+ from which it originated but never to h^c, whereas the mutant h^ch^b reverts to h^c but never to h^+.

The existence of an indefinite number of loci governing mutation in the *r* loci has also been established. Hershey (92) found that the mutation may be phenotypically expressed as *s* (strong lysis inhibition), wild-type, *w* (weak lysis inhibition), and *r*, arranged in order of descending potencies of lysis inhibition. Analyses of these four phenotypes and their mutants showed that rapid mutations always occur in the direction of weaker lysis inhibition (from *s* and wild-type to *w* and *r*) whereas slower mutations, selected by natural competition during serial passage, are always directed toward stronger lysis inhibition (reversion from *r* and *w* to *s* or wild-type).

In a more recent report, Hershey & Rotman (94) found that all the independently arising *r* mutants of T2H are genetically different as expressed by the fact that bacteria infected with any two such *r* mutants liberate viral progeny containing a measurable proportion of lysis-inhibiting types in contrast to the liberation of *r* particles only for bacteria infected with either of the *r* mutants alone. The amount of wild-type obtainable from intercrosses (the term intercross, as used by Hershey & Rotman, refers to one step growth experiments with mixed multiple infection) of different *r* mutants serves to classify the numerous *r* mutants into two classes so that class A crossed with class B yields rather constantly about 15 per cent of wild-type. Crosses between different members within

the same class yield proportions of wild-type characteristic for each pair; the amount of wild-type obtainable in this manner varies between 0.5 and 8 per cent. There is some evidence suggestive of linkage, for the amounts of wild-type appearing in any three possible crosses between different *r* mutants are approximately additive, though intercrosses among sets of four *r* mutants cannot be strictly interpreted in terms of four independent transfer frequencies. Evidently the *r* mutants differ also in their rate of spontaneous reversion to wild type and in the kind of reversion as evidenced by the production of viruses which are either genetically identical with or different from the wild-type.

The biochemical mutants described by Anderson (35) consisted of T4 virus particles that no longer exhibited cofactor requirements and, hence, were adsorbable directly on susceptible host cells; these probably arose by mutation of the cofactor-requiring types during intracellular multiplication in *E. coli* B. Delbrück (36) has described two additional deficient forms of T4 virus, one of which required tryptophane as cofactor, but whose adsorption could be inhibited by traces of indole, while the second type required both tryptophane and calcium ion for adsorption and was likewise inhibited by indole.

A phenomenon suggestive of an induced mutation in the *r* locus giving rise to liberation of new types of viruses has been reported (67). In order to elicit this phenomenon it was necessary to resort to mixed infections with pairs of the even-numbered viruses in which one of the pair was used in the wild-type form and the other in the *r*-type form. For example, in a mixed infection with the viruses $T2r^+$ and $T4r$ the plating on B/2 gave plaques the majority of which had mottled halos, indicating that most of the bacteria had liberated a mixture of wild-type and *r*-type particles capable of attacking B/2. The wild-type particles, therefore, represented a new type, created probably during the mixed infection through a modification of $T4r$ to $T4r^+$ under the influence of $T2r^+$. The experiments also demonstrated that mutations could occur from wild-type (r^+) to *r*-type or from *r*-type to wild-type, but only if one of the infecting types was wild-type while the other was *r*-type. These results may also be interpreted as a possible transfer of genetic materials, thus confirming the observations of Hershey (92) which showed that bacterial cells infected with h^+r and $h^ch^br^+$ viruses give rise to a series of new types.

Reactivation

Earlier investigations (37) had shown that ultraviolet-inactivated T2 viruses were still adsorbable onto host cells; such irradiated viruses, however, failed to grow within the infected bacteria, presumably because inactivation of the phage particles had occurred when one of their essential units or genes suffered a lethal hit. In a more recent study, Luria (95) has demonstrated that when two or more ultraviolet-irradiated viruses attached themselves to the same bacterial cell, reactivation of the inactive viruses may ensue. It appears that reactivation results because two or more inactive particles are able to pool their remaining active genes to give rise to active virus; only when the same genes have been inactivated in both phages will virus reproduction fail to occur.

The phenomenon of reactivation occurs only for the large-particle phages, T2, T4, T6, T5, and their mutants. The reaction is partially specific in that a concentrated suspension of one phage can generally not reactivate the particles of another irradiated phage; however, an important exception is that cross-reactivation can occur between suspensions of the even-numbered phages, T2, T4, and T6 (95).

The likelihood of reactivation or recombination of genes was assumed to be a function of the total number of genes which can be calculated from the experimental data. Luria (95) has estimated that the minimum number is at least 30 to 50 for some of these phages.

The same phenomenon has been reported by Watson (96) after irradiation of phage with x-rays; quantitatively, however, there is less chance for x-irradiated phages to undergo reactivation. The reason for this is probably correlated with the possibility that one x-ray hit produces, on the average, more lethal mutations than is the case following ultraviolet irradiation.

Luria & Dulbecca (97) have concluded that the genetic transfers involved in the reactivation are not reciprocal exchanges occurring prior to multiplication, for they found that bacteria infected with one inactive irradiated T2r^+ and one inactive T2*r* particle give rise to a mixture of active T2r^+ and T2*r*.

The mechanism of transfer in reactivation appears to be a transfer of units "by infection" rather than an exchange of portions of gene strings, thus differing from that of crossing over (95).

These findings appear to be at variance with Hershey & Rotman's data (94). However, subsequent investigations by Hershey & Rotman (98) have revealed new information on this problem. These workers found that genetic recombinations may occur between two viruses, *h* (mutant with altered host range) and *r*1, *r*7, or *r*13 (different plaque mutants), differing by two independent mutations. Thus, nearly all mixedly infected bacteria yield both parental types of virus, *h* and *r*, as well as two recombinants, *hr* and wild-type. Closely linked systems such as $h \times r13$ result in a very small number of genetic exchanges in contrast to less closely linked systems. Hershey & Rotman have suggested an extension of Luria's concept whereby genetic exchanges occur either by reassortment of independently multiplying chromosome-like structures or by something resembling crossing over between homologous pairs, depending on the structural relationships between the genetic factors involved.

Attempts to Demonstrate Cellular Components Reacting with Phage

In addition to the work accomplished with the T set of phages, considerable research has been carried on by Krueger and his co-workers, using the K strain of *Staphylococcus aureus* and the K race of phage. They first demonstrated that the rate of phage production is not determined by the rate of cellular reproduction and were able to establish conditions under which phage was formed while bacterial division was entirely suppressed (99, 100, 101). These observations were confirmed by Northrop (102).

Later Krueger & Mundell (103) found that the active growth of staphylococci followed by treatment to induce a resting state conferred upon the organisms the capacity to raise the activity titre of phage some tenfold. Reproduction of the activated staphylococci was not a factor, for the reaction was conducted in Locke's solution at 5°C. and seemed to take place very rapidly (104). A normal 18 hr. culture did not produce such an increment.

This state of activation is readily abolished by exposing the cocci to the action of iodoacetic acid (105), the photodynamic activity of methylene blue (106), sonic vibrations (107), or to heat (104). These various treatments can be so graded that lethal effects on the organisms are avoided. A protein appears to be involved in the case of thermal inactivation, for the reaction possesses the high temperature coefficient characteristic of protein denaturation.

A better understanding of the reaction occurring between staphylococci and the homologous phage has followed the application of both the plaque count and the activity assay method as analytical procedures (108). Of particular interest, mostly so far as the present discussion is concerned, are the sharp increments in activity titre which occur during the periods when the plaque count is constant. Within a matter of a few minutes the [total phage]—mostly intracellular—will increase from 17- to 56-fold. Later, after the rise in activity titre is complete, redistribution of the newly

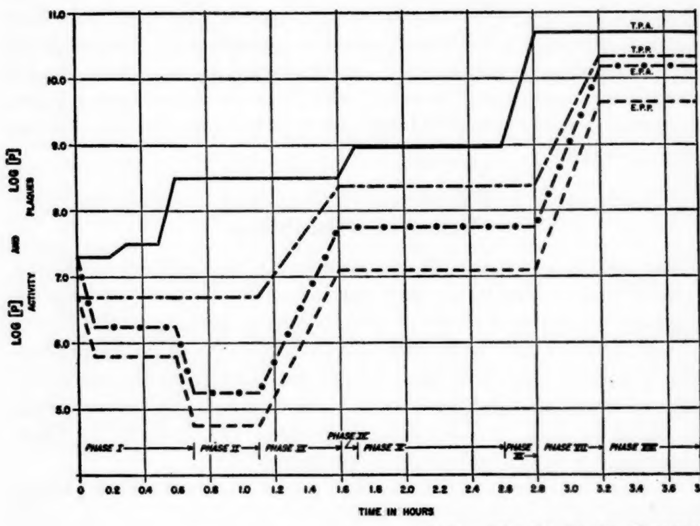

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FIG. 1

formed phage takes place as evidenced by corresponding rises in the total plaque count. The general relationships involved are shown in Fig. 1. These data suggest that intracellular phage formation does not take place at a constant rate but is completed in a brief period just before lysis occurs, in keeping with the evidence secured by Latarjet (58) by means of roentgen irradiation.

Krueger and his co-workers have pursued the working hypothesis that rapidly metabolizing cocci elaborate some sort of intracellular components which react with phage to form more phage.

This concept is compatible with the experimental evidence cited above, but, at the same time, the observed phenomena can be explained in other ways. Probably the only acceptable proof of this theory would be isolation of the material or materials participating in the reaction with phage and responsible for the rise in titre. Many attempts have been made in this direction. Some slight increases in activity titre were reported with ultrafiltrates of growing staphylococcal suspensions but the results were irregular (109). Filtrates of cell preparations lysed by penicillin gave no increases nor did cells mechanically disrupted by exposing them to sonic vibrations. It was not until 1948 that statistically significant increases in plaque counts were obtained by using actively growing cells lysed by lysozyme (110). These experiments are open to two major objections; the increases in phage plaque titre obtained conceivably could be due to dispersion of phage aggregates into smaller units or to conversion of inactive phage into an active form.

Experiments have been performed to test these possibilities. If dispersion of phage or reactivation of inactive phage were the basis of the reaction, serial dilutions of phage in filtrate should soon exhaust either the number of aggregates or the original store of inactive phage. While the range of phage concentrations which can be used is narrow and this in turn limits the extent of the serial dilutions, it has been possible to perform experiments in which six serial dilutions were made with measurable increases in [phage] in each step. For example, in one such experiment eight serial dilutions of phage in filtrate were made using initial phage concentrations of 2.5×10^6 and a dilution factor of $\frac{1}{2}$. The percentage yields for individual steps varied between 136 to 220 per cent. By dilution of original phage, the end titre should be 1.9×10^4; the actual titration value was 1.2×10^5 or 6.3 times the calculated concentration.

Further efforts were made to exclude the possible role of an inactive phage fraction in producing the observed increments in titre by using stock phages prepared in such a manner that the theoretical maximal yield of phage per cell was obtained. When these lysates were employed in testing the cell-free filtrates of lysozyme-lysed staphylococci, the usual increases in plaque counts were obtained (110).

Kingkade, Huff & Behrens (111) have reported studies with lysozyme from which they conclude that no increases occur in experiments of the type described above. In the presence of lyso-

zyme the reaction between phage and susceptible cells is modified in the direction of decreasing the burst size.

It appears to the writer that the working hypothesis whereby formation of phage can result from the use of cellular enzyme systems is sound, although the experimental evidence now available relative to this point is not conclusive and further work must be done before the issue can be settled.

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